JOURNAL

OF

BACTERIOLOGY

VOLUME 32

BALTIMORE, MD.
1936

CONTENTS

No. 1, July, 1936

A Study of Methods for the Determination of Reducing Sugars in Bacterial Cultures. Colorimetric Methods. Dorothea Klemme and Charles F. Poe 1

Adsorption of Bacteria in Salt Lakes. L. Rubentschik, M. B. Roisin and F. M. Bieljansky 11

Studies on Tetanus Toxoid. III. Antitoxic Response in Guinea Pigs Immunized with Tetanus Alum-precipitated Toxoid Followed by Tetanus Spores. F. G. Jones and W. A. Jamieson 33

Studies of Streptococci. IV. Resistance of Enterococci. George H. Chapman 41

The Bactericidal and Photochemical Properties of Irradiated Cod Liver Oil and an Ozonide of Olive Oil. Franklin A. Stevens 47

Effectiveness of Hot Hypochlorites of Low Alkalinity in Destroying *Mycobacterium tuberculosis*. S. M. Costigan 57

Hydrogen Sulfide Production as a Differential Test in the Colon Group. Reese Vaughn and Max Levine 65

Ultra-Violet Irradiated Carbohydrates and Bacterial Growth. John George Baumgartner 75

Clostridium botulinum Type C in Relation to Duck Sickness in the Province of Alberta. R. M. Shaw and Gretta S. Simpson 79

The Life Cycle of the Organism Causing Yeast Meningitis. Ramona L. Todd and Walter W. Herrmann 89

A Study of Variation in Hemolytic Streptococci from Scarlet Fever and Erysipelas. II. Comparative Virulence, Carbohydrate Fermentation, Toxin Production of the S and R Strains. Protective Power of S and R Vaccines. Reversion. Sophie Spicer, assisted by Emily L. Bloom and Mary F. Gonshorek 105

Proceedings of Local Branches of the Society of American Bacteriologists. North Central, Northern California-Hawaiian, Connecticut Valley, and Society of Illinois, Branches 115

No. 2, August, 1936

Curdled Blood. Simply Prepared Fluid Medium for Cultivation of Anaerobes. E. Murray Pullar 131

Semisolid Media for Cultivation and Identification of Sporulating Anaerobes. R. S. Spray 135

Essential Growth Factors for Propionic Acid Bacteria. I. Sources and Fractionation. E. L. Tatum, W. H. Peterson and E. B. Fred 157

Essential Growth Factors for Propionic Acid Bacteria. II. Nature of the Neuberg Precipitate Fraction of Potato. E. L. Tatum, H. G. Wood and W. H. Peterson 167

Bacteriological Approach to Problem of Interconversion of Hexoses in Alkaline Solutions. A. G. Wedum 175
Physiological Studies on Rhizobium. V. The Extent of Oxidation of Carbonaceous Materials. O. R. Neal and R. H. Walker 183
Bacteriology of *Bacillus mycoides* with Reference to Effect on Dissociation and Transmission by Spores. I. M. Lewis and Gordon Worley 195
Specificity of Dye in Crystal-violet Agar Reaction of Staphylococci. George H. Chapman 199
Studies on Cultural Requirements of Bacteria. VIII. Utilization of Glutamic Acid by Diphtheria Bacillus. J. Howard Mueller 207
Indol Tolerance of Certain Strains of Colon-Aerogenes Group. Ralph L. France 211
Phenol Coefficient as Measure of Practical Value of Disinfectants. J. C. Varley and G. F. Reddish 215
Identification of Hemagglutinin of Jack Bean with Concanavalin A. James B. Sumner and Stacey F. Howell 227
Proceedings of Local Branches of the Society of American Bacteriologists. Central Pennsylvania Branch 239

No. 3, SEPTEMBER, 1936

The Spread of Tularemia through Water, as a New Factor in Its Epidemiology. S. P. Karpoff and N. I. Antonoff 243
The Occurrence of Salmonella, Oranienburg Type, in an Infection of Quail. Philip R. Edwards 259
Studies of Freshwater Bacteria. III. Quantitative Aspects of the Direct Microscopic Method. Arthur T. Henrici 265
The Action of Hexamethylenetetramine on Members of the Colon and Aerogenes Groups. Charles F. Poe and J. Howard Williamson 281
An Atypical Meningococcus Isolated from Cerebrospinal Fluid in the Calcutta Meningitis Epidemic of 1934–35. Benode Behari Sen 293
The Influence of Optical Activity on the Utilization of Tryptophane for Growth by the Diphtheria Bacillus. Lyle C. Bauguess 299
The Fermentation Reactions of *Erysipelothrix rhusiopathiae*. A. E. Deem and Charles L. Williams 303
The Estimation of Bacterial Populations with the Aid of a Photoelectric Densitometer. L. G. Longsworth 307
A Study of the Characteristics of Variants Derived from Single Cells of *Escherichia coli*. John C. Torrey and Elizabeth Montu 339
Proceedings of Local Branches of the Society of American Bacteriologists, Central New York State Branch 355

No. 4, OCTOBER, 1936

Sonic Energy as a Lethal Agent for Yeast and Bacteria. T. D. Beckwith and C. E. Weaver 361
A Critical Study of Some of the Growth-promoting and Growth-inhibiting Substances Present in Brilliant-green Bile Medium. C. N. Stark and L. R. Curtis 375

Increased Growth and Gas Production by Escherichia-Aerobacter Organisms in Brilliant Green Bile Medium Containing Sodium Formate. C. N. Stark and L. R. Curtis 385
Substitutes for Potassium in Metabolism of the Lowest Fungi. Otto Rahn .. 393
The Serologic Agglutination of the Obligate Anaerobes *Clostridium paraputrificum* (Bienstock) and *Clostridium capitovalis* (Snyder and Hall). Marshall L. Snyder 401
Mechanism of the Non-specific Serum Agglutination of the Obligate Anaerobes *Clostridium paraputrificum* and *Clostridium capitovalis*. Marshall L. Snyder 411
Correlative Studies of Microscopic and Plate Methods for Evaluating the Bacterial Population of the Sea. Margaret Hotchkiss and Selman A. Waksman 423
Changes in the Electrokinetic Potential of Bacteria at Various Phases of the Culture Cycle. Laurence S. Moyer 433
Proceedings of Local Branches of the Society of American Bacteriologists, Northern California-Hawaiian Branch 465

No. 5, November, 1936

Metabolism of Various Types of Sugars by S and R forms of Pneumococcus. Philip Finkle 473
Growth and Fermentation of Bacteria Near Their Minimum Temperature. Milton J. Foter and Otto Rahn 485
Sugar Alcohols. VI. Utilization of Sugar Alcohols and Their Anhydrides by Various Microörganisms. K. Pierre Dozois, C. Jelleff Carr, John C. Krantz, Jr., Frank Hachtel and Frances F. Beck 499
Absorption of Staphylococcus Bacteriophages. Morris L. Rakieten, Tony L. Rakieten and Simon Doff 505
Influence of the Composition of the Medium on the Metabolism of Some Slow-lactose-fermenting Bacteria of Intestinal Origin. A. D. Hershey and J. Bronfenbrenner 519
Some Chemical Factors Influencing Growth and Pigmentation of Certain Microörganisms. M. S. Kharasch, E. A. Conway and W. Bloom 533
Studies on Hemolytic Streptococci. III. *Streptococcus equi* and Related Strains. Alice C. Evans 541
Spontaneous Transformation of Pneumococcus Type V to Type II. L. A. Barnes and Eleanor C. Wight 557
Bacterial Growth at Constant pH. Apparent Oxidation-reduction Potential, Acid Production, and Population Studies of *Lactobacillus acidophilus* under Anaerobic Conditions. Lewis G. Longsworth and D. A. MacInnes 567
Proceedings of Local Branches of the Society of American Bacteriologists, New York City Branch 587

No. 6, December, 1936

Attempts to Increase the Heat Resistance of Bacterial Spores. Fred T. Williams 589

Glycerol and Carbohydrate Utilization by *Mycobacterium tuberculosis*. A. G. Wedum 599
The Growth of Microörganisms on Media Exposed to Ultra-violet Radiations. Edward L. Pratt 613
A Lytic Action of Certain Strains of Hemolytic Streptococci on Fresh Sterile Kidney and Other Tissues. Beatrice Carrier Seegal and David Seegal 621
A Comparative Study of Hemolytic Streptococci Isolated from the Throats of Residents of New Orleans and New York. Paul Teiger and Beatrice C. Seegal 631
The Preparation of Silicic Acid Jellies for Bacteriological Media. John H. Hanks and Robert L. Weintraub 639
The Pure Culture Isolation of Ammonia-oxidizing Bacteria. John H. Hanks and Robert L. Weintraub 653
Proceedings of Local Branches of the Society of American Bacteriologists, Eastern Pennsylvania and Central Pennsylvania Branches 671
Index 675

A STUDY OF METHODS FOR THE DETERMINATION OF REDUCING SUGARS IN BACTERIAL CULTURES

COLORIMETRIC METHODS

DOROTHEA KLEMME AND CHARLES F. POE

Division of Sanitary Chemistry, Department of Chemistry, University of Colorado, Boulder, Colorado

Received for publication, April 12, 1935

In a study of the metabolic products formed by the colon and aerogenes groups of bacteria in sugar media, it became necessary to determine accurately the amounts of residual sugar in the culture-media. A search of the literature revealed that only two methods had been used for the determination of reducing sugars in bacteriological media. Stiles, Peterson, and Fred (1926) tried the Shaffer-Hartmann method (1921) and found it to be satisfactory for the determination of glucose in bacteriological media, both before and after clarification with lead acetate. Magee and Smith (1930), however, found the Shaffer-Hartmann method to be inaccurate when applied to media containing meat infusions. These investigators likewise found the Benedict copper method (1926) to give unsatisfactory results.

Consequently, the authors decided to investigate the various colorimetric methods as to their availability for use in the analysis of reducing sugars present in broths used for the growth of bacterial cultures. The results of this investigation are reported in the present communication.

The medium used for the growth of the different cultures contained 8 grams of Bacto-Nutrient broth and 10 grams of glucose to the liter. The reaction was adjusted to a pH of 7, and no calcium carbonate was added. The cultures used were members of the *Escherichia* and *Aerobacter* groups of bacteria.

The medium was placed in 500-cc. florence flasks, 200 cc. to the flask. After sterilization for twenty minutes at a pressure of 15

pounds, the medium was stored at 4°C. until used. Inoculations were made from actively growing cultures of each organism. The medium used for growing these cultures was of the same composition as that used in the flasks. All incubations were carried out at 37°C.

TABLE 1

Amounts of glucose found after organisms were grown in 1 per cent glucose medium
(Results expressed in milligrams per 100 cc. medium)

TIME OF GROWTH	LEWIS-BENEDICT	SUMNER	KINGSBURY	DINITRO-PHENOL	FOLIN-WU	BENEDICT	FOLIN
			a. *E. coli* (average 7 strains)				
hours							
Original	1,070	1,004	998	1,009	1,031	984	992
12	921	894	898	893	896	873	882
24	863	802	800	810	816	801	814
48	826	791	782	794	806	784	792
72	884	696	702	698	716	689	702
			b. *E. communior* (average 7 strains)				
Original	1,063	1,009	989	1,002	1,016	994	1,001
12	876	826	822	830	826	831	846
24	852	798	774	803	796	788	821
48	828	790	758	781	786	769	800
72	796	756	726	740	751	722	752
			c. *A. aerogenes* (average 7 strains)				
Original	1,082	1,013	992	1,009	1,003	980	1,005
12	806	762	700	772	782	732	789
24	560	465	440	472	465	458	488
48	255	198	164	210	188	186	186
72	95	24	13	26	39	26	36

The colorimetric methods used were the Lewis-Benedict (1915) method as modified by Willaman and Davidson (1924), Benedict (1928), Folin-Wu and Folin (1929), Sumner (1925), Kingsbury (1927), and the 2:4 dinitrophenol method proposed by Poe and Edson (1932).

Table 1a contains the average results obtained for the determination of glucose after 7 different strains of *Escherichia coli*

had been separately grown in the medium. Table 1b gives similar results when 7 strains of *Escherichia communior* were used; and table 1c lists the results for 7 strains of *Aerobacter aerogenes*. Duplicate determinations were run on the medium for some of the cultures. Satisfactory checks were obtained in each case.

In a number of cases, it was noted that the results obtained by certain methods were abnormally high. This fact was especially

TABLE 2

Reducing substances present in peptone broth during the growth of E. coli and A. aerogenes

(Results expressed in milligrams per 100 cc. medium)

TIME OF GROWTH	LEWIS-BENEDICT	SUMNER	KINGSBURY	DINITRO-PHENOL	FOLIN-WU	BENEDICT	FOLIN
			E. coli (average 3 strains)				
hours							
Original	42	0	0	0	8	0	5
12	50	0	0	0	12	0	7
24	52	0	0	0	21	0	12
48	62	0	0	0	24	0	14
72	58	0	0	0	20	0	12
96	63	0	6	0	22	0	14
			A. aerogenes (average 3 strains)				
Original	120	0	0	0	10	0	12
12	134	0	0	0	14	0	16
24	202	0	0	0	20	0	20
48	152	0	0	0	22	0	24
72	150	0	8	0	26	0	30
96	156	0	10	0	32	0	28

true with the Lewis-Benedict method. A series of experiments were made to determine whether or not any reducing substances were present in the original medium or were formed when the organisms were grown in the broth to which no sugar had been added. The broth used was prepared by adding 8 grams of Bacto-Nutrient broth to 1 liter of water. The results of these experiments are given in table 2.

It will be noted that the Lewis-Benedict method showed

considerable reducing substances when *Escherichia coli* and *Aerobacter aerogenes* were grown in plain broth. The Folin and Folin-Wu methods showed some reducing substances. Little or no increase in reducing substances was shown by the following methods: Sumner, Kingsbury, Benedict, and the Dinitrophenol. Apparently, enough reducing substances are originally present in the broth and are formed during the growth of the bacteria to cause some error when the Lewis-Benedict, Folin, and Folin-Wu methods are used.

A point in question concerned the amount of glucose which could be recovered by analysis when different amounts of this sugar were added to the medium. The medium which originally contained 1 per cent glucose was checked by analysis with each of the colorimetric methods. After 0.5 per cent glucose had been added, similar determinations were made. Tests were also made by adding 0.5 per cent glucose to the cultures after fermentation had taken place for various lengths of time.

The average results for ten determinations are given in table 3. The organisms used in determinations 1 to 5 were *E. coli*; in determinations 6 to 10, *A. aerogenes* (table 4). Magee and Smith (1930) could not recover, quantitatively, added glucose when measured with the Benedict method. The average results as given in table 3 show a fairly satisfactory recovery of glucose for all of the methods except the Folin method. The data in this table do not present the whole story. In table 4 is listed the percentage recovery for the determination with each of the ten cultures. The average percentage recovery for Benedict's method (95.8) is fairly satisfactory; however, the recovery given in the individual determinations varies greatly. For this method, there is a spread of over 32 per cent—from 83.0 to 115.8 per cent. The spreads for the determinations by the other methods are not so great as in Benedict's method and are as follows: Folin 22.0 per cent; Lewis-Benedict, 19 per cent; Kingsbury, 13.2 per cent; Dinitrophenol, 7.4 per cent; Folin-Wu, 6.2 per cent; and Sumner, 5.2 per cent. The Folin-Wu, Sumner, and Dinitrophenol methods, therefore, gave a satisfactory recovery of glucose.

Check determinations in the same sample of culture medium gave fairly satisfactory results for all the methods except the

TABLE 3

Recovery of added glucose

(Results expressed in milligrams per 100 cc. medium*)

METHOD	GLUCOSE FOUND		TOTAL THEORETICAL AMOUNT OF GLUCOSE	AMOUNT OF ADDED GLUCOSE RECOVERED	PER CENT RECOVERY
	Original medium	After 0.5 gram added			
Lewis-Benedict	729	1,235	1,229	506	101.2
Sumner	788	1,293	1,288	505	101.0
Kingsbury	718	1,197	1,218	479	95.8
Dinitrophenol	732	1,237	1,232	505	101.0
Folin-Wu	749	1,232	1,249	483	96.6
Benedict	721	1,200	1,221	479	95.8
Folin	732	1,173	1,232	441	88.2

* The data are the average of the results obtained when glucose was added to the media in which ten different organisms were grown.

TABLE 4

Percentage recovery of added glucose from media in which ten different cultures were grown

NUMBER OF DETERMINATION	LEWIS-BENEDICT	SUMNER	KINGSBURY	DINITRO-PHENOL	FOLIN-WU	BENEDICT	FOLIN
1	111.4	103.0	100.4	104.4	99.4	115.8	102.2
2	98.0	98.8	94.4	98.5	99.6	109.0	95.2
3	97.0	103.8	98.0	99.6	93.4	96.4	88.8
4	106.8	100.4	93.4	99.9	93.8	86.6	88.4
5	98.2	103.8	88.2	102.3	94.4	83.0	82.4
6	111.0	101.2	101.0	104.6	97.8	91.2	90.4
7	92.4	100.6	89.6	97.7	96.2	100.4	89.4
8	101.6	98.6	94.0	103.4	97.6	86.4	80.2
9	99.0	98.8	101.4	102.3	96.8	104.4	84.4
10	96.6	101.4	97.6	97.2	96.6	84.8	80.2
Average....	101.2	101.0	95.8	101.0	96.6	95.8	88.2

Benedict. In this method the two check determinations would sometimes show as much as 0.075 gram difference on the basis of 0.50 gram of sugar. For the Folin and Lewis-Benedict method,

the maximum difference was 0.03 gram; for the Kingsbury and Folin-Wu, 0.02 gram; and for the Sumner and Dinitrophenol, 0.015 gram.

The effect of dilution was tried on the recovery of added glucose. With each method, dilutions of 1:1, 1:2, and 1:4 were tried. The dilution within the above-mentioned limits did not affect the accuracy of the methods.

All experiments up to this point were made on media which had not been clarified. A number of the cultures, especially the aerogenes cultures, produced considerable cloudiness in the broth. Several agents were used to clarify the media before analyses were made for glucose. The results obtained in the analysis of glucose in the clarified medium were compared with the amount of glucose obtained in the unclarified medium.

A brief summary of the methods of clarification follows: Starch: Ordinary potato starch was washed several times with water and then dried. To 100 cc. of the diluted medium (1:10) were added 3 grams of starch. The mixture was shaken for ten minutes and then filtered. Carbon, Norite: To 100 cc. of the diluted medium was added 1 gram of norite decolorizing carbon, and this mixture was allowed to stand with frequent shaking for ten minutes. The mixture was then filtered. Fuller's Earth: Three grams of fuller's earth were added to 100 cc. of the diluted medium and allowed to stand for ten minutes with frequent shaking. The mixture was then filtered. Mercuric Nitrate: To 10 cc. of the medium was added 1 cc. of mercuric nitrate solution (10 grams of mercury and 8 cc. concentrated HNO_3 were heated on a hot plate, cooled, and added to 8 cc. concentrated HNO_3. To this were added 3 cc. of 5 per cent NaOH, after which the solution was made up to 100 cc. and stored in the dark). The mixture was shaken frequently during ten minutes, after which it was diluted to 100 cc. and filtered. The filtrate was made slightly alkaline to litmus with sodium bicarbonate. The mixture was filtered again. Alumina Cream: To 10 cc. of the medium were added about 50 cc. of water and 5 cc. of alumina cream,[1] and the whole mixture was diluted to 100

[1] Prepared according to Leach. See Food Inspection and Analysis, page 810, 4 ed., 1920.

cc. The mixture was shaken and allowed to stand ten minutes; then it was filtered. *Lead Subacetate Solution:* One cubic centi-

TABLE 5

The effect of clarifying agents on the determination of glucose in medium in which organisms are grown

(Results expressed in milligrams per 100 cc. medium)

	No clarification	Folin & Saker	Lloyd's	Lloyd's	Mercuric nitrate solution	Uranium acetate	Lead subacetate solution	Lead subacetate dry
a. A. aerogenes (average of 3 strains)								
Lewis-Benedict	798	796	778	799	558	991	794	748
Sumner	787	755	769	753	568	772	757	754
Kingsbury	758	770	759	778	668	775	774	781
Dinitrophenol	775	789	759	757	566	770	758	757
Folin-Wu	799	775	754	778	573	781	775	799
Benedict	736	741	722	754	672	742	722	739
Folin	772	775	771	799	681	753	755	758
b. B. coli (average of 3 strains)								
Lewis-Benedict	918	927	918	930	694	782	925	918
Sumner	892	927	907	899	669	896	896	892
Kingsbury	888	881	884	901	738	902	896	891
Dinitrophenol	901	896	894	896	654	895	918	892
Folin-Wu	908	895	908	912	587	919	899	906
Benedict	865	854	848	899	551	872	881	872
Folin	891	885	891	892	576	881	878	884
c. B. communior (average of 3 strains)								
Lewis-Benedict	890	899	884	895	595	798	884	879
Sumner	882	856	861	873	562	865	872	865
Kingsbury	870	868	857	858	675	880	876	864
Dinitrophenol	882	860	854	874	591	878	870	864
Folin-Wu	868	836	840	870	463	868	871	860
Benedict	842	826	818	844	491	849	862	841
Folin	870	865	855	896	558	868	879	858

meter of 30 per cent solution of Horn's anhydrous lead subacetate was added to 10 cc. of medium and diluted to 100 cc. The solution was allowed to stand ten minutes and was then filtered.

the maximum difference was 0.03 gram; for the Kingsbury and Folin-Wu, 0.02 gram; and for the Sumner and Dinitrophenol, 0.015 gram.

The effect of dilution was tried on the recovery of added glucose. With each method, dilutions of 1:1, 1:2, and 1:4 were tried. The dilution within the above-mentioned limits did not affect the accuracy of the methods.

All experiments up to this point were made on media which had not been clarified. A number of the cultures, especially the aerogenes cultures, produced considerable cloudiness in the broth. Several agents were used to clarify the media before analyses were made for glucose. The results obtained in the analysis of glucose in the clarified medium were compared with the amount of glucose obtained in the unclarified medium.

A brief summary of the methods of clarification follows: Starch: Ordinary potato starch was washed several times with water and then dried. To 100 cc. of the diluted medium (1:10) were added 3 grams of starch. The mixture was shaken for ten minutes and then filtered. Carbon, Norite: To 100 cc. of the diluted medium was added 1 gram of norite decolorizing carbon, and this mixture was allowed to stand with frequent shaking for ten minutes. The mixture was then filtered. Fuller's Earth: Three grams of fuller's earth were added to 100 cc. of the diluted medium and allowed to stand for ten minutes with frequent shaking. The mixture was then filtered. Mercuric Nitrate: To 10 cc. of the medium was added 1 cc. of mercuric nitrate solution (10 grams of mercury and 8 cc. concentrated HNO_3 were heated on a hot plate, cooled, and added to 8 cc. concentrated HNO_3. To this were added 3 cc. of 5 per cent NaOH, after which the solution was made up to 100 cc. and stored in the dark). The mixture was shaken frequently during ten minutes, after which it was diluted to 100 cc. and filtered. The filtrate was made slightly alkaline to litmus with sodium bicarbonate. The mixture was filtered again. Alumina Cream: To 10 cc. of the medium were added about 50 cc. of water and 5 cc. of alumina cream,[1] and the whole mixture was diluted to 100

[1] Prepared according to Leach. See Food Inspection and Analysis, page 610, 4 ed., 1920.

cc. The mixture was shaken and allowed to stand ten minutes; then it was filtered. Lead Subacetate Solution: One cubic centi-

TABLE 5

The effect of clarifying agents on the determination of glucose in medium in which organisms are grown

(Results expressed in milligrams per 100 cc. medium)

	NO CLARIFICATION	FULLER'S EARTH	CARBON	STARCH	MERCURIC NITRATE SOLUTION	ALUMINA CREAM	LEAD SUBACETATE SOLUTION	LEAD SUBACETATE DRY
a. *A. aerogenes* (average of 3 strains)								
Lewis-Benedict	798	796	778	790	558	491	794	788
Sumner	767	755	749	763	568	772	767	764
Kingsbury	768	770	759	776	608	775	774	781
Dinitrophenol	775	769	759	757	566	770	768	767
Folin-Wu	769	776	764	778	573	781	774	769
Benedict	736	741	732	764	462	742	732	739
Folin	772	775	771	759	481	763	765	758
b. *E. coli* (average of 3 strains)								
Lewis-Benedict	918	927	918	930	694	742	925	918
Sumner	892	887	897	899	609	896	896	892
Kingsbury	888	881	884	891	738	902	896	891
Dinitrophenol	900	896	894	886	654	895	903	892
Folin-Wu	908	895	898	912	587	919	899	906
Benedict	865	854	848	869	551	872	881	872
Folin	891	885	890	892	576	881	878	884
c. *E. communior* (average of 3 strains)								
Lewis-Benedict	890	896	884	895	505	798	884	879
Sumner	862	856	861	873	562	865	872	865
Kingsbury	870	868	857	858	675	880	876	864
Dinitrophenol	862	860	854	874	591	878	870	864
Folin-Wu	868	836	840	870	463	868	871	860
Benedict	842	826	818	844	491	849	862	841
Folin	870	865	855	886	558	868	879	858

meter of 30 per cent solution of Horn's anhydrous lead subacetate was added to 10 cc. of medium and diluted to 100 cc. The solution was allowed to stand ten minutes and was then filtered.

The filtrate, after being treated with anhydrous sodium carbonate until no more lead precipitated, was filtered. Dry Lead Subacetate: To 100 cc. of the diluted medium were added 3 grams of Horn's anhydrous lead subacetate. The mixture was freed from the excess lead with sodium carbonate.

The results obtained after using the different above-mentioned clarifying agents are given in table 5.

The results in table 5 show that starch, fuller's earth, norite carbon, and lead subacetate are satisfactory as clarifying agents for bacteriological media. A solution of mercuric nitrate when used to clarify the media causes a very low percentage of glucose to be recovered. It was not satisfactory with any of the colorimetric methods. Alumina cream proved to be a satisfactory clarifying agent in all of the methods except the Lewis-Benedict.

A study of table 5 together with the previous tables indicates that the Sumner, the Folin-Wu, and the Dinitrophenol methods are the most accurate ones for the determination of residual sugar in bacterial cultures. The Lewis-Benedict method gives results which are too high. In some cases the Kingsbury and the Benedict methods give results slightly too low. The recovery of added glucose with the Lewis-Benedict, the Kingsbury, the Benedict, and the Folin methods is not satisfactory. A number of the clarifying agents are suited for use when the colorimetric methods are to be used for determining the sugar content. All of the agents tried except mercuric nitrate and alumina cream are satisfactory with all of the methods. Apparently, however, accurate results may be obtained without clarification.

SUMMARY

1. Seven colorimetric methods have been investigated for use in determining reducing sugars in bacterial culture-media.

2. The accuracy of the sugar determination, when clarifying agents are used, has been investigated.

REFERENCES

BENEDICT, S. R. 1926 Jour. Biol. Chem., **68**, 759.
BENEDICT, S. R. 1928 Jour. Biol. Chem., **76**, 457.
FOLIN, O. 1929 Jour. Biol. Chem., **82**, 83.

KINGSBURY, F. B. 1927 Jour. Biol. Chem., **75**, 241.
LEWIS, R. C., AND BENEDICT, S. R. 1915. Jour. Biol. Chem., **20**, 61.
MAGEE, M. C., AND SMITH, H. G. 1930. Jour. Bact., **19**, 125.
POE, C. F., AND EDSON, F. G. 1932. Jour. Ind. and Eng. Chem., Anal. Ed., **4**, 300.
SHAFFER, P. A., AND HARTMANN, A. E. 1921 Jour. Biol. Chem., **45**, 349, 365.
STILES, H. R., PETERSON, W. H., AND FRED, E. B. 1926 Jour. Bact., **12**, 427.
SUMNER, J. B. 1925 Jour. Biol. Chem., **65**, 393.
WILLAMAN, J. J., AND DAVIDSON, F. R. 1924 J. Agric. Research, **28**, 479.

ADSORPTION OF BACTERIA IN SALT LAKES

L. RUBENTSCHIK, M.B. ROISIN AND F. M. BIELJANSKY
Microbiological Laboratory, State University of Odessa, U. S. S. R.

Received for publication January 25, 1936

INTRODUCTORY

The adsorption of bacteria by porous and powder-like substances has for a long time been the subject of research. It was shown by Krüger (1899) that the number of bacteria present in water may be reduced by addition of coke, sand, clay, brick-dust, magnesium oxide and other powder-like substances. The adsorbent action is much greater in the case of substances with a low specific gravity than with those whose specific gravity is high. The results were confirmed by a number of other investigators. According to Eisenberg (1918), the degree of adsorption of bacteria depends upon their lipoid contents which is higher in the cells of the gram-positive bacteria than in those of gram-negative forms. However, there is no sharp dividing line between these two groups, as regards adsorptive capacity: some gram-positive species (*Corynebacterium diphtheriae*) are only faintly adsorbed, while some gram-negative species (*Serratia marcescens*) are well adsorbed.

The problem of absorption of bacteria is of particular interest in soil investigation (Chudiakow, 1925–26; Dianowa and Woroshilowa, 1925). The degree of adsorption depends upon the size of the soil particles. The smaller their size the higher is the percentage of adsorbed bacteria. This holds true, however, only up to a certain limit beyond which there is a sharp decline in the degree of adsorption. Soil particles which do not exceed 0.27μ do not adsorb bacteria at all. On the contrary, Karpinska (1925) has shown that such particles may be adsorbed by large bacteria, as by the chains of *Bacillus mycoides*. The same phe-

nomenon of reversibility of adsorption was observed by Bechhold (1918) in experiments with white clay and other substances.

The results of our own work show that the adsorption of bacteria by liman mud exhibits a number of characteristic features; namely, dependence of the degree of adsorption not upon the concentration but upon the absolute number of bacteria; difference in adsorption of living and of dead bacteria; the presence in *Serratia marcescens* of a zone of maximum adsorption. However, it must be pointed out that, in general, Gibb's equation is only rarely fulfilled. In most cases it is accompanied by phenomena which complicate the picture and sometimes even disguise the adsorption itself. It is for this reason that, in the case of bacteria taken up by mud, we only provisionally employ the term adsorption. Later, however, after a more detailed study of the phenomena involved, it will be necessary to raise the question whether it really may be regarded as true adsorption.

EXPERIMENTAL

Methods

Various sediments are found at the bottom of salt lakes or limans, in the vicinity of Odessa, the most interesting of which may be considered the black mud. The latter is a complex product which owes its origin to various microbiological processes and contains a great quantity of microbes more than 3 billions in 1 gram of some samples. Nothing is known regarding the condition of the microbes in the mud, i.e., whether they are there in a free state or adsorbed by the mud. The capacity of black mud and other ground sediments of salt lakes for the adsorption of bacteria remained to be elucidated. There has been so far published only a brief statement (Sweshnikowa, 1926) to the effect that when mud of the Tembukan lake was shaken up with a bacterial suspension the number of bacteria in the latter decreased.

The method used in the following investigations can be outlined briefly as follows: Several loopfulls of a two- or three-day old agar slant culture of bacteria were introduced into a sterile flask containing sterilized tap-water. The bacterial mass was

then carefully distributed in the water. Since some small lumps of bacteria usually remained, it was necessary to filter the suspension through sterile filter paper. Two identical test-tubes received 10-cc. portions of this suspension. One test-tube received a definite amount of the adsorbent while the other remained as a control. Both tubes were shaken for the same length of time and the adsorbent mud allowed to settle. Usually the shaking lasted one minute while it took half an hour for the mud to settle. The number of bacteria was determined by diluting 10,000 or 100,000 times, and plating the final dilution on meat-peptone agar.

The accuracy of this method was determined in a preliminary experiment. To twenty-five test-tubes each containing 2 grams of mud were added 10-cc. portions of an aqueous suspension containing 160 millions of cells of *Serratia marcescens*. After one minute's shaking and thirty minutes settling of the mud the percentage of cells adsorbed in each test-tube was calculated. The data thus obtained were subjected to calculation by the method of variation, according to the equation (Sapehin, 1926):

$$M = \pm \sqrt{\frac{\Sigma v^2}{n} - M^2} \cdot \sqrt{\frac{1}{n-1}}$$

As a result the percentage of cells adsorbed was found to be: 96.4 ± 1.5; p (index of accuracy) = 1.55 per cent.

In those cases in which the plating method could not be employed (for instance, when the degree of adsorption of dead bacteria was to be determined), the direct microscopic method was used. For this purpose one drop of the above described diluted bacterial suspension was placed in a counting chamber (American standard Haemocytometer). The count was made in twenty-five large squares. It was, however, necessary to take into consideration the fact that the adsorbent, namely the mud or any other of the ground sediments, contains many bacteria, some of which may have been washed off while the adsorbent was shaken with water. As a control, the adsorbent was also shaken with 10 cc. of sterilized tap-water. Ten cubic centimeters of a suspension of *Serratia marcescens* contained a large enough number

of cells to give in one large square of the counting chamber, on the average, 100 bacteria. After shaking with 2 grams of mud for one minute and allowing the mud to settle for thirty minutes, the suspension contained on the average 14 bacteria in one square. Two grams of the same mud when the mud was shaken up for one minute with 10 cc. of sterilized water, and the mud allowed to settle for thirty minutes, gave 6 bacteria in one large square. It follows, therefore, that, of the remaining 14 bacteria, only 8 cells were *Serratia marcescens*. Hence the percentage of adsorption was 92. Table 1 illustrates the adsorption of 4 different bacteria by marine mud.

TABLE 1

Adsorption of different bacteria by the mud of the Kuyalnik liman

	NAME OF ORGANISM							
	S. marcescens		*B. vulgatus*		*B. mycoides*		*Sarcina lutea*	
	a	b	a	b	a	b	a	b
Number of bacteria in 10 cc. of the suspension, in millions:								
Before adsorption	125.4	301.4	31.4	150.0	59.5	210.0	25.1	130.0
After adsorption	1.0	14.2	3.4	25.5	0.4	14.7	0.24	7.5
Number of adsorbed bacteria, in millions	124.4	287.2	28.0	124.5	59.1	195.3	24.86	122.5
Percentage of adsorption	99.2	95.3	89.2	83.0	99.3	93.0	99.4	95.0

The next experiment (table 2) deals with the adsorption by the mud of different bacteria isolated from the liman water (nos. 1 and 2) and mud (nos. 3 to 7). The adsorption of the latter was much greater than of the former.

It was further found that bacteria isolated from the liman water are relatively little adsorbed and wash off easily while bacteria found in the mud are adsorbed in great quantity and few of them may be washed off with ease.

Besides black mud we have also investigated the adsorption capacity for bacteria of the other types of liman sediments. The following results were found to represent the percentage of adsorption of *S. marcescens:*

Non-plastic black-gray mud	68.0–79.1
Gray plastic mud	45.8–84.8
Black (young) mud	71.2
Black sand	39.5–61.9
Gray sand[1]	19.3–20.5
Grayish-black sand	21.0–22.4

It is evident from these data that the various types of ground sediments differ in their adsorption capacity for *S. marcescens*. The highest adsorption capacity took place with the plastic black mud and the lowest with the gray sand.

In all of the experiments referred to above the samples of the sediments investigated were taken at a depth of 10 to 15 cm. below the surface of the liman bottom. In one case only was the

TABLE 2

Adsorption of the bacteria of the liman water and mud

	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6	NO. 7
Number of bacteria in 10 cc. of the suspension (in millions):							
Before adsorption	135.5	34.0	133.3	78.5	86.3	109.9	78.5
After adsorption	45.6	7.2	0.2	0.7	0.9	0.25	0.5
Number of adsorbed bacteria (in millions)	89.9	26.8	133.1	77.8	85.4	109.65	78.0
Percentage of adsorption	66.6	79.8	99.8	99.2	99.0	99.8	99.4

adsorption capacity of deeper layers of the sediments also determined. The surface layer was black sand; at a depth of 1.25 m. gray non-plastic mud mixed with sand was found, while at a depth of 2.25 m. gray plastic mud was present. The percentage of adsorption of *S. marcescens* was found to be:

Surface layer	26.2
Layer at a depth of 1.25	39.8
Layer at a depth of 2.25	96.2

Desorption of S. marcescens from mud

Frei and Erismann (1922) reported that the number of bacteria in a suspension was greater after prolonged shaking of the latter

[1] The sand deposits of the liman contain some admixture of clay and of organic substances.

with sand than after short-time shaking; the difference in the results was explained by partial desorption. Dianowa and Woroshilowa (1925) have also observed partial desorption of bacteria inoculated in soil. In the case of *S. marcescens* we succeeded in washing off 13 millions of cells out of 219.8 millions inoculated in 2 grams of mud, i.e., 6 per cent.

The following experiment was performed in order to determine the influence of the length of time allowed for washing off on the degree of desorption. Into each of four test-tubes containing 2 grams of mud, 1-cc. portions of a suspension of *S. marcescens* containing 800.7 millions of bacterial cells, was added. After careful stirring, 9 cc. of sterilized water were added to each test-tube; one of the test-tubes was shaken 5 seconds, one 30

TABLE 3

800.7 millions of cells of S. marcescens in 2 grams of mud

TIME	NUMBER OF BACTERIA WASHED OFF	PERCENTAGE OF DESORPTION
seconds	*millions*	
5	5.0	0.6
30	23.2	2.9
60	46.2	5.8
300	49.6	6.2

seconds, one 60 seconds and one 300 seconds. The results presented in table 3 show that the percentage of bacteria desorbed increased from 5 to 60 seconds. However, if washing was continued for a longer period, the number of bacteria washed off remained unchanged, the difference being within the limits of the error of the experiment.

Has the mud a toxic effect?

In connection with the plating method, used for the bacterial count, the possibility suggested itself that the decrease in the number of bacteria in the suspension after the latter had been shaken with mud, may have been due not to adsorption but to a toxic effect of the mud. To test the correctness of this assumption, the following experiment was performed. Into each of three

test-tubes containing 2 grams of mud, 1-cc. portions of the same suspension of *S. marcescens* were introduced and carefully mixed with the mud. Nine cubic centimeters of sterilized water were added to one test-tube after five minutes, to the second after thirty minutes and to the third after sixty minutes, and the test-tubes were shaken for one minute.

The following number of cells of *S. marcescens* was washed off:

After 5 minutes contact with the mud	3.3 millions
After 30 minutes contact with the mud	3.7 millions
After 60 minutes contact with the mud	3.1 millions

At the same time the number of cells of *S. marcescens* found in the mud was determined after they had been there for five, thirty and sixty minutes respectively. The number of cells was found to be in all cases the same, namely 60 millions per gram. It is thus evident that the mud did not show in sixty minutes any toxic action.

The influence of bacterial numbers on adsorption

Two grams of mud were shaken for one minute with 10 cc. of a suspension of *S. marcescens* which contained 4.8 million, 55 million, 110 million, 220 million and 395 million cells respectively. The length of time allowed for the settling of the mud was in all cases thirty minutes.

The results obtained (table 4) show that, beginning with 110 million cells the percentage of adsorption decreased with an increase in the number of bacteria in the suspension. When, however, the number of cells introduced was below 110 millions, the percentage of adsorption also decreased. In another experiment in which the number of bacteria in the suspension was 17.3 millions, 171.2 millions, 387.6 millions, 1,031 millions and 1,719 millions, respectively, the percentage of adsorption proved to be 88, 97.1, 90 and 71.

A characteristic peculiarity of adsorption is the fact that a relatively greater quantity of the substance in question is adsorbed from dilute solutions than from more concentrated ones. So far as our experiments are concerned, this rule proved to hold true, only when 10 cc. of the suspension contained not less than

110 to 171 millions of bacteria. In cases where the number of bacterial cells was smaller, the percentage of adsorption did not increase, as might have been expected according to this rule, but dropped. Thus, in the experiments with *S. marcescens*, a zone of maximum adsorption was evident within the limits between 110 millions and 171 millions of bacteria present in the suspension, while on both sides of this zone the degree of adsorption decreased.

TABLE 4

The influence of the number of bacterial cells

	A	B	C	D	E
Number of bacteria in 10 cc. of suspension (in millions):					
Before adsorption	4.8	55.0	110.0	220.0	385.0
After adsorption	1.0	2.7	1.2	9.4	46.2
Number of bacteria adsorbed (in millions)	3.8	52.3	108.8	110.6	338.8
Percentage of adsorption	81.0	95.0	98.9	95.7	88.0

The influence of the quantity of the adsorbent

In the following experiments, the quantity of adsorbent, i.e., of mud, was varied within the limits of 0.1 to 5 grams. Ten-cubic centimeter portions of a bacterial suspension containing 100 millions of cells of *S. marcescens* were added to test-tubes containing varying quantities of mud. All of the test-tubes were shaken for one minute and thirty minutes were allowed for the mud to settle. Table 5 shows that the percentage of adsorption was 80.5 when 0.1 gram of mud was used, 88.7 in the case of 0.5 gram, 94.7 with 2 grams and 99.2 with 5 grams.

Influence of acid treatment of mud

In order to show how a change in chemical composition of the mud influenced its adsorbing properties, mud was subjected to the action of a 10-per-cent solution of hydrochloric acid. Some of the colloids ($FeS \cdot H_2O$, $Al(OH)_3$) were dissolved. At the same time the usual alkaline reaction of the mud changed to

acid. To 2 grams of the altered, as well as of the normal mud, 10 cc. of a suspension of *S. marcescens* containing 135 million cells were added. The data given in table 6 show that the normal mud adsorbed 98 per cent of the bacteria added, while the one treated with HCl adsorbed only 20 per cent. It is evident from this result that the treatment of the mud with hydrochloric acid caused a sharp decline in its adsorption capacity.

TABLE 5

The influence of the quantity of mud

	QUANTITY OF MUD			
	0.1 gram	0.5 gram	2.0 grams	5.0 grams
Number of bacteria in 10 cc. of the suspension (in millions):				
Before adsorption	100	100	100	100
After adsorption	19.5	11.3	5.3	0.8
Number of bacteria (in millions)	80.5	88.7	94.7	99.2
Percentage of adsorption	80.5	88.7	94.7	99.2

TABLE 6

The influence of hydrochloric acid on the adsorption capacity of the mud

	UNTREATED MUD	MUD TREATED WITH HCl
Number of bacteria in 10 cc. of the suspension (in millions):		
Before adsorption	135.0	135
After adsorption	2.7	108
Number of bacteria adsorbed (in millions)	132.3	27
Percentage of adsorption	98.0	20

Exchange adsorption

It is well known that the liman mud contains a great number of diverse bacteria, some of which grow on meat-peptone agar. A part of these latter organisms are washed off (desorbed) from the mud when the latter is shaken with water. If now the water used for shaking contains some bacteria capable of being adsorbed by mud, then, a simultaneous adsorption of these bacteria and desorption of some bacteria contained in the mud should

occur. The following experiment shows the quantitative relation between the bacteria adsorbed and those desorbed. Mud (2 grams) was shaken up for one minute with 10 cc. of sterilized tap-water. As a result, it was found that 300,000 bacteria were washed off the mud. In another test-tube 2 grams of the same mud were shaken with 10 cc. of tap-water which contained 4.8 millions of cells of *S. marcescens*. In this latter case it was found that 4,175 millions of cells of *S. marcescens* had been adsorbed by the mud while 725,000 cells of other bacteria were washed off. Hence, it is clear that the adsorption of 4,175 millions of cells of *S. marcescens* caused an increase of the number of bacteria desorbed to 725,000 − 300,000 = 425,000, i.e., for every 10 cells of *S. marcescens* adsorbed there was washed off the mud one cell more than in the experiment with sterilized water. It is evident from these results that an exchange adsorption of bacteria is going on in the mud.

The survival of adsorbed bacteria in the mud

In order to determine the influence of adsorption on the viability of bacteria, the following experiment was carried out. Sterilized black mud (5 grams) was shaken with 10 cc. of a suspension containing 100 million of cells of one of the following species, taken from our laboratory collection: *Serratia marcescens*, *Bacillus mycoides*, *Bacillus vulgatus* and *Sarcina lutea*. As soon as the mud had settled, the suspension was poured off and the test-tubes with the mud closed with cotton plugs and placed in a dark moist-chamber at room temperature. Every five days, small lumps of the mud were taken from each test-tube with a platinum loop and smeared on the surface of solidified meat-peptone agar in Petri dishes. At the beginning of the experiment 50 to 75 mgm. of mud were taken for each sowing. Later, however, 1 gram of mud was always used. Whenever the bit of mud contained cells still capable of growth, colonies were found to have formed around it in a few days. As a result of these experiments, it was established that the adsorbed bacteria retained their viability for the following time periods:

	days
Ser. marcescens	40
S. lutea	55
B. vulgatus	>80
B. mycoides	>80

Influence of adsorption on the morphological, cultural and biochemical properties of bacteria

Serratia marcescens, *Bacillus vulgatus* and *Sarcina lutea* were used on this experiment. An aqueous suspension of these bacteria was shaken with sterilized mud for one minute, and after the latter had settled down, the suspension was poured off and the test-tubes closed with cotton plugs and kept in the dark at room temperature. The adsorbed bacteria remained in the mud for twenty-five days; they were then again isolated (by the plating method), inoculated on various culture media (meat-pepton broth, meat-pepton agar, meat-pepton gelatin, milk, potatoes) and their morphological, cultural and bio-chemical properties studied. Similar studies have been also made of cultures of normal, not-adsorbed bacteria which had been kept on meat-pepton agar for twenty-five days.

Only the most significant changes may be noted: *Sarcina lutea* and *Serratia marcescens* that had been adsorbed by mud were no longer capable of liquefying gelatin. *Serratia marcescens* did not coagulate milk and formed no pigment or very little of it. In the case of *Sarcina lutea* the size of the cells decreased somewhat and the majority of the cells were not arranged in packets but in twos or fours. These deviations proved to be temporary only, for after several re-inoculations under standard conditions the original properties of the cultures reappeared. Thus, the adsorption of saprophytic bacteria by mud for twenty-five days did not lead to any profound changes in their morphological, cultural and biochemical properties.

Similar results have also been obtained in experiments with pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*) introduced into liman mud (Rubentschik, Hoicherman and Reich, 1932).

The influence of adsorption on the activity of microbiological processes in the mud

As already mentioned above, Dianowa and Woroshilowa have recorded a decrease of CO_2 production by bacteria adsorbed by the soil. Chudiakow (1926) assumed that this might be due to the absence of oxygen in the soil colloids, as established by Russel and Appleyard (1915).

The influence of adsorption on the processes of nitrification, oxidation of thiosulphate and sulphate reduction were determined in these experiments.

a. Nitrification. It is so far known (Rubentschik, 1929) that only the bacteria of the first phase of nitrification have been found in the Kuyalnik liman. Morphologically and culturally they appear to be identical with Winogradsky's *Nitrosomonas europea*, but differ from all known strains of the latter species in their obligatory halophily.

A culture of this liman species in Omelianski's medium made up with liman water (5° Baumé) has been used. After strong stirring of the liquid culture 4 cc. were transferred to a flask containing 16 cc. of sterilized liman water. After thorough mixing, the bacterial suspension was divided into two test-tubes. To one, 2 grams of oxidized sterilized mud[2] were added: the test-tube was shaken for one minute and left for thirty minutes to allow the mud to settle. The number of nitrifying bacteria in the suspensions of both test-tubes was then determined. The dilution method was employed, using Omelianski's medium for *Nitrosomonas*, made up with liman water. The flasks were placed in the thermostat at a temperature of 28° to 30°C. for thirty days. The bacteria were found to be adsorbed to the extent of 99 per cent.

The oxidation of ammonia was determined in the medium containing bacteria adsorbed by mud as well as in cultures which

[2] As was shown previously, neither oxidation nor sterilization of the mud has any notable effect on its adsorption capacity. Since the nitrifying bacteria are obligatory aerobes, their activity in the liman is possible only in the oxidized surface-layer of the mud. It was for this reason that in our experiment with *Nitrosomonas* we employed oxidized mud.

had not been adsorbed. For this purpose two flasks containing 28 cc. of sterile medium and 2 cc. of a *Nitrosomonas* culture were used. Two cubic centimeters of the same culture were placed in a test-tube containing 8 cc. of Omelianski's medium and 2 grams of oxidized and sterilized mud. After shaking the test-tube for one minute its contents were poured into the second flask, to which about 18 cc. of sterile medium were added, so that the surface level of the suspension appeared to be the same in both flasks. After the flasks had been in the thermostat for thirty days at a temperature of 28° to 39°C., 49 mgm. of nitrite were found to have formed in the first flask and only 18 mgm. in the second. In a later experiment in which the same method was employed, 57 and 21 mgm. respectively of nitrite have been obtained.

b. Oxidation of thiosulphate. It was shown by Saslawsky (1927) that the thiosulphate-oxidizing bacteria of the Kuyalnik liman are obligatory halophiles. In our experiments on adsorption we used a culture of these bacteria on Beijerinck's medium (with thiosulphate), made up with liman water. The methods employed for the determination of the percentage of adsorption of these bacteria by sterilized, oxidized mud as well as of the activity of thiosulphate oxidation by adsorbed and by not-adsorbed bacteria were exactly the same as in our experiment with *Nitrosomonas*.[3] As a result, it was found that, out of 100 million bacteria inoculated, 90 millions were adsorbed by 2 grams of mud. Accordingly the percentage of adsorption was 90. After twenty days, the medium containing bacteria that had not been adsorbed by mud had 165 mgm. of thiosulphate oxidized, while 163 mgm. were oxidized in the medium with bacteria which had not undergone adsorption. In another analogous experiment the quantity of oxidized thiosulphate amounted to 126 and 121 mgm. respectively. These results proved that adsorption has no effect on the activity of the thiosulphate-oxidizing bacteria of the liman.

[3] The only difference in the technique employed in the two cases was that, while the quantity of oxidized thiosulphate was determined by titration with 0.01 N I, the quantity of nitrite formed was found by titrating with 0.01 N $KMnO_4$.

c. Sulphate reduction. The sulphate-reducing bacteria of the Odessa limans are halophiles (Chait, 1924; Rubentschik, 1928). In the experiments described below, a culture of bacteria of the Kuyalnik liman in Beijerinck's medium made up with liman water was used. The degree of adsorption of these bacteria was determined in the following way. After active stirring of the culture in Beijerinck's medium, 2 cc. were transferred to a flask containing 18 cc. of sterilized liman water. The contents of the flask were thoroughly stirred and 10 cc. were poured into each of two test-tubes. To one of the latter, 2 grams of black mud were added and, after one minute's shaking and thirty minutes settling, the number of bacteria in the suspensions of both test-tubes determined by the dilution method. The material to be inoculated was poured into small wide-necked jars, 50 cc. in volume, filled with Beijerinck's medium prepared with liman water. The jars were closed with ground stoppers, care being taken to leave no air bubbles. Anaerobic conditions were thus obtained, indispensable for the growth of sulphate-reducing bacteria. The jars were kept in the thermostat at a temperature of 28° to 30°C. for a month and subsequently their contents were titrated with 0.01 NI. This experiment showed that out of 10 millions of cells 9 millions had been adsorbed by the mud. The percentage of adsorption, accordingly, was 90. For the comparative determination of the activity of sulphate reduction 2 cc. of a culture of the sulphate-reducing bacteria were inoculated in a flask containing 18 cc. of liman water and after thorough stirring the bacterial suspension was poured in equal parts (i.e., 10 cc. each) into two test-tubes. To one of the test-tubes 2 grams of black mud were added and after shaking for one minute the contents of both test-tubes were poured into two small jars. Since hydrogen sulphide (2 to 15 mgm.) was present in the mud placed in one of these jars, it was necessary for the sake of obtaining like conditions of anaerobiasis in the other jar without mud, to add the same quantity of hydrogen sulphide to the latter. Both jars were then filled to the top with Beijerinck's medium, closed with ground stoppers and kept in the thermostat at a temperature of 28° to 30°C. After twenty-five days it was found

that 20.3 mgm. of H_2S were formed in the jar containing bacteria adsorbed by mud and only 12.4 mgm. in the jar without mud. In another analogous experiment 23.8 and 13 mgm. respectively of H_2S were formed. These results show that the sulphate reduction was more active in the medium with adsorbed than in that with not-adsorbed bacteria.

DISCUSSION

Rawitsh (1930) found that the fraction of the mud which is not soluble in a 10-per-cent solution of HCl takes up cations (K, Na, Mg) as well as basic dyes, while neither anions (Cl, SO_4) nor acid dyes are adsorbed. The fact that this fraction of mud which contains mainly compounds bearing the negative charge (silicic acid) adsorbs substances that bear the positive charge would seem to indicate that the adsorption capacity of the mud may depend upon the magnitude and the sign of the electric charge of its component parts. It has been shown that when mud is treated with a 10-per-cent solution of HCl the result is a sharp decline in its adsorption capacity for bacteria. In this case the hydrochloric acid dissolves these ingredients of the mud which have the positive charge ($FeS \cdot H_2O$, $Fe(OH)_2$, $Fe(OH_3)$, $Al(OH)_3$). On the other hand it is known that living bacteria usually have a negative charge (Kraus and Uhlenhuth, 1923; Buchanan and Fulmer, 1928; Rubinstein, 1932). The conclusion thus seems to be justified that the adsorption of bacteria by mud depends upon electric phenomena taking place at the boundary between the surfaces of mud particles and those of the bacteria cells. It is possible, however, that besides electric forces some other factors are here at work, for instance the magnitude of the surface tension, the degree of the moistening capacity of the surface of the bacterial cell, its adhesion capacity to the mud, etc. Here further work is necessary.

There are some contradictory statements in the literature regarding the influence of adsorption on bacteria. According to Karpinska (1925), bacteria adsorbed by the soil are in a condition of depression and even perish in part. This is the reason why no bacterial growth is observed when soil containing adsorbed

bacteria is inoculated in a culture medium. Chudiakow and Karpinska assume that the forces underlying bacterial adsorption suffice to destroy the bacterial cell. Frei and Erismann (1922) also find that adsorption of bacteria by sand filters leads to a depression and even to partial dying off of the bacteria. Bechhold and Schlossberger (1923), on the contrary, assume that adsorption as such has no injurious action on bacteria. Friedberger and Kumagai (1918) found that when some bacteria die as a result of adsorption, this is due to the mechanical injury which they sustain from the sharp points of the adsorbent. But whenever bacteria, previously adsorbed by animal coal, kaolin and other indifferent adsorbents are plated out, the authors could in each case record growth on suitable culture media. Our own experiments show that adsorbed bacteria retain their vitality in the mud for a long time, and when subsequently transferred into suitable culture media, they grow there and call forth certain processes. That is why we are inclined to think that adsorption alone, as such, has no influence on bacteria. It might, however, result in some collateral phenomena which affect the life processes of bacteria.

In this connection the following possibilities may now be considered. In case the metabolic products of the bacteria are not taken up by a certain adsorbent, adsorption may, *ceteris paribus*, unfavorably affect the bacteria. For it must be kept in mind that while bacteria, when free, may occupy all of the space of the culture, they are, when adsorbed, localized only in that part of the medium which is occupied by the adsorbent. In the latter case, however, around every adsorbed cell there should be a zone of more highly concentrated toxic metabolic products than around free cells, especially, if the latter are motile. If, however, the metabolic products are adsorbed, the effect on the adsorbed bacteria may be a favorable one.

This assumption is supported by some data recorded in the literature. Thus, according to Söhngen (1913), the addition of various colloids (ferric oxide, animal coal a. o.) to a culture of Urobacteria stimulates the decomposition of urea, as these colloids adsorb the ammonia formed. The growth of yeast and the

formation of alcohol is also increased by colloidal substances, which possibly adsorb the noxious products of metabolism (Reinhard and Obrastzowa, 1935; Plewako and Kunsburgsky, 1932). Just how nitrogen-fixing bacteria are activated by colloids is not yet clear; it is possible, however, that in this case the adsorption of metabolic products may play its part; adsorption may change the conditions of the nutrition of bacteria. Since the latter when adsorbed occupy a part only of the medium, the nutritive substances of the latter may at some time become exhausted and the normal metabolism will depend upon the rapidity of diffusion of nutritive substances from the remaining part of the medium. Some nutrients may, besides, be taken up by the adsorbent employed and thus be unavailable for the bacteria. Furthermore, what has been said regarding the conditions of nutrition of adsorbed and free bacteria applies also to their respiration, for the access of oxygen to every bacterial cell may be altered by adsorption. The action of adsorption is accordingly, an indirect one and its effect, while favorable in one way may be detrimental in other respects. The final result depends upon the total environmental conditions of the case and upon the bacterial species. It is evident from these considerations that no generalizations can be made regarding the influence of adsorption on bacteria and that a special study is needed in each particular instance to determine this influence.

In our investigation only three microbiological processes which take place in salt lakes have been considered, and yet it was possible to ascertain that the adsorption of bacteria by ground sediments has a marked influence on the degree of activity of some bacteria, lowering it in some cases (nitrification) and increasing it in other cases (sulphate reduction). *On this basis adsorption may be regarded as an ecological factor which should be taken into account in any study of the reciprocal action between microbes and the environment in salt lakes.* Our experiments have shown that the various types of ground sediments differ in their adsorption capacity. The idea thus suggests itself of employing the method of bacterial adsorption for the study of the ground sediments in salt lakes. In the classification of these sediments,

paleontological, petrographic, physicochemical and other data have so far been considered. It is possible that the study of the bacterial adsorption capacity might contribute new and interesting material that could be made use of for the same purpose.

It is a well known fact that microbes play an important part in the formation of black mud in salt lakes. There are various microbiological processes going on in the limans and other salt lakes, as a result of which simple decomposition products of animal and plant bodies as well as more complex compounds are formed. These substances impregnate the clayish-sandy skeleton, giving rise to the black, plastic, greasy mass—black mud. One of the factors which influences the microbiological process is, as was shown in the preceding pages, the adsorption of bacteria. It follows from this that the latter deserves the attention of balneologists in connection with the problem of the formation and conservation of therapeutic mud.

Little is known so far, concerning the distribution of bacteria in salt lakes. The question regarding the factors which may control the formation of bacterial benthos and bacterial plankton is entirely in the dark. Data regarding the bacterial species inhabiting the bottom and the water of the limans are very meagre and a mere beginning has so far been made. Yet the data presented above in regard to bacterial adsorption would seem to point the way of approach to the problem regarding the factors that influence the distribution of bacteria in these lakes. It was shown by our experiments that liman mud contains bacteria, the degree of adsorption of which is higher and of desorption lower than that found in bacteria isolated from liman water. Hence the conclusion that *the bacterial benthos in salt lakes should consist of easily adsorbable species while the bacterial plankton should contain species that have a low degree of adsorption by the ground sediments.* While it is clear that the local distribution of the bacteria on the bottom and in the body of the water depends not on one but on many factors, it appears from this investigation that in adsorption we have found one of these factors.

It is, however, clear that the bacterial population of the upper layer of the ground sediments and also of the water of the limans cannot be invariably the same. Every year, especially when the

snow is thawing, small rivers and ravines carry into the limans a great number of various species of bacteria differing not only with regard to their systematic position but also in the degree of their capacity for adsorption. Some of them perish under the specific conditions of life in the limans while others adapt themselves. Owing to reciprocal adsorption which we have noted, the upper layer of the sediments adsorbs some and at the same time releases other species. The bacterial flora of this layer should thus be subject to periodic changes in regard to both quantity and species.

Some of the drainage water from the sewage fields of Odessa flows into the Chadjibey liman. The Coli-titer of this water usually varies within the limits from 0.1 to 0.01. A great number of *Escherichia coli* are thus constantly carried into the liman. Analyses, however, have shown that with increasing distance from the sewage fields a very marked decrease of the number of this bacterial species in the liman water is noted. Thus at a distance of about 100 m. from the sewage fields the Coli-titer appears to be the same (from 50 to 100) as in various other parts of the liman. The question now arises, what becomes of these colon bacilli? The possibility of their rapid death may be left out of consideration, for there is experimental evidence for the long survival of this species in liman water, (Rubentschik, Hoichermann and Reich 1932). The adsorption capacity of the ground sediments of the Chadjibey liman for this species was found to be high. The conclusion is thus fully justified that the disappearance of *E. coli*, brought into the liman by the drainage water is due to its being adsorbed. Owing to shallowness of the shore water and to frequent tides in this lake the various layers of the water and also the upper layer of the ground sediments easily intermix. And this should result in the adsorption of *E. coli*. It is thus evident that adsorption plays an important part in the processes of self-purification of the water in salt-lakes.

SUMMARY

1. The ground sediments of the limans (salt lakes) are capable of adsorbing bacteria.

2. The various types of sediments differ in their adsorption

capacity for the same bacterial species. The adsorption capacity of the black, plastic mud is especially high.

3. The degree of adsorption of various species of bacteria by a certain type of sediment differs according to the species.

4. Bacteria adsorbed by mud may be partly desorbed.

5. Bacteria isolated from mud are more readily adsorbed and less readily desorbed than bacteria isolated from liman water.

6. The percentage of adsorption decreases when the quantity of bacteria in the suspension is increased. In the case of *Serratia marcescens*, however, a zone of maximum adsorption has been observed when 10 cc. of the suspension contain from 110 million to 171 millions of cells. The degree of adsorption declines on both sides of this maximum zone.

7. The percentage of bacteria adsorbed increases with the increase (up to a certain limit) of the quantity of the adsorbent (mud).

8. The adsorption capacity of black mud is changed neither by oxidation nor by sterilization in the autoclave. A sharp decline of the adsorption capacity, however, results when the mud is treated with a 10-per-cent solution of HCl.

9. The degree of adsorption depends not upon the quantity of the liquid medium of the concentration of bacteria but upon their absolute number.

10. The same quantity of bacteria are adsorbed from a suspension made up with liman water as from a suspension prepared with tap-water.

11. The degree of adsorption is higher for living bacteria than for those killed by high temperature.

12. When two bacterial species are adsorbed simultaneously the one may affect the degree of adsorption of the other.

13. An exchange adsorption takes place in the liman mud, the adsorption of some species being accompanied by desorption of other species from the mud.

14. Adsorbed bacteria may survive in the mud for a long time without any profound changes in their morphological, cultural and bio-chemical properties being noted.

15. Adsorption may affect the life processes of the bacteria adsorbed by the mud, the activity of some (nitrification) being

lowered while that of others, on the contrary, is increased (sulphate reduction).

16. The differences in the degree of adsorption and of desorption of bacteria inhabiting the ground sediments and the liman water suggest the assumption that adsorption may play some part in the differentiation between bacterial plankton and benthos forms of the limans.

17. The adsorption of bacteria plays a part in the self-purification of the water in salt-lakes.

REFERENCES

Bechhold, H. 1918 Kolloid-Zeitschr., **23**, 35.

Bechhold, H., und Schlossberger, H. 1923 Handbuch der mikrobiol. Technik, Herausgeg. von Kraus und Uhlenhuth, **2**, 1413.

Buchanan, R. E., und Fulmer, E. I. 1928 Physiology and Biochemistry of Bacteria, **1**, 312.

Chait, S. 1924 Journal nautsch.-issled. Kafedr w Odesse (Russian), **1**, Nr. 10–11, 32.

Chudiakow, N. N. 1925–1926 Nautschno-agron. Journal, **2**, 742; Centrbl. f. Bakt., Abt. 2, **68**, 345; Pedology, **2**, 98; Selsko-Chosaistwenaja Microbiologia, p. 215.

Dianowa, E. W., and Woroshilowa, A. A. 1925 Nautschno-agron. Journal, **2**, 520.

Eisenberg, P. 1918 Centrlbl. f. Bakt., Abt. 1, Orig., **81**, 72.

Frei, W., und Erismann, H. 1922 Centrlbl. f. Bakt., Abt. 1, Orig., **88**, 306.

Friedberger, E., und Kumagai, T. 1912. Zeitsch. f. Immunitätsforsch., **13**, 127.

Karpinska, N. S. 1925. Nautschno-agron. Journal, **3**, 587.

Kraus, R., und Uhlenhuth, P. 1923 Handbuch der mikrob. Technik, **2**, 1419.

Krüger, W. 1889. Zeitsch. f. Hyg., **7**, 135.

Plewako, E. A., and Kunsburgsky, F. M. 1932 Brodilnaja Promishlenost (Russian), Nr. 7, 17.

Rawitsh, M. 1930. Iswestia Inst. Phys.-Chim. Analisa (Russian), **4**, 384.

Reinhard, A., et Obrastzowa, V. 1935 Bolletino dela sezione italiana Soc. Inter. di Microbiol., **7**, Fasc. VIII–IX, 331.

Rubentschik, L. 1928 Centrlbl. f. Bakt., Abt. 2, **73**, 483.

Rubentschik, L. 1929 Centrlbl. f. Bakt., Abt. 2, **77**, 1.

Rubentschik, L., Hoichermann, D., and Reich, G. 1932 Trudi Wseukr. Balneo-Physio-Therapewt. Int., **1**, 12.

Rubentschik, L., and Hoichermann, D. 1935. Microbiology (Russian), **4**, 403.

Rubinstein, D. L. 1932 Phis.-Chim. Osnowi Biologii (Russian), p. 229.

Russel, E. J., and Appleyard, A. 1915 Jour. Agric. Sci., **7**, 1.

Sapehin, A. A. 1926 Variatsina Statistika (Ukrain), p. 89.

Saslawsky, A. S. 1927 Centrlbl. f. Bakt., Abt. 2, **72**, 236.

Söhngen, N. L. 1913 Centrlbl. f. Bakt., Abt. 2, **38**, 636.

Sweshnikowa, I. A. 1926 Trudi Balneol. Instit. Kawkas. Mineral. Wod (Russian), **3**, 165.

STUDIES ON TETANUS TOXOID

III. ANTITOXIC RESPONSE IN GUINEA PIGS IMMUNIZED WITH TETANUS ALUM-PRECIPITATED TOXOID FOLLOWED BY TETANUS SPORES

F. G. JONES AND W. A. JAMIESON

Lilly Research Laboratories, Indianapolis, Indiana

Received for publication February 4, 1936

When an individual has been given one or more injections of tetanus toxoid and, at a later date, is injured and possibly infected with *Clostridium tetani*, what further precautions should be taken: (1) Administer antitoxin? (2) Give another injection of tetanus toxoid to accelerate the production of antitoxin? (3) Give no further treatment, depending on his present immunity to take care of the infection?

The original idea in administering tetanus toxoid was to avoid the use of antitoxin. We have shown (Jones and Moss, 1936; Jones, 1936) that another injection of toxoid at the time of injury will quickly increase the antitoxin content twentyfold.

This study was undertaken to ascertain whether a huge dose of tetanus spores would accelerate the production of additional antitoxin. Owing to the fact that there has been some difficulty in producing tetanus consistently in animals, we tried various methods.

Gye and Cramer (1919) found that the ionizable salts of calcium injected together with the washed spores of *Cl. tetani* or of certain other anaerobes led to the development of the corresponding infections in their typical and fatal forms, while the washed spores alone showed no tendency to develop in the tissue after inoculation. The chlorides of sodium, potassium, ammonium, strontium, or manganese had no such effect.

Later; Fildes (1927, 1929 a, 1929 b) showed that in the sub-

cutaneous tissues of the living guinea pig the oxidation-reduction potential (Eh) at pH 7.0, is about +0.12 volt, while tetanus spores will not germinate readily at a potential more oxidizing than +0.01 volt at this pH. He further showed that the injection of solutions of calcium chloride led to the production of localized areas of oxygen deficiency, thus providing conditions suitable for the germination of tetanus spores.

In our first experiment 1 cc. of a semisolid agar culture of *Cl. tetani* was mixed with 9 cc. of a 10 per cent solution of calcium chloride. Three guinea pigs were injected with this mixture as follows:

GUINEA PIG NUMBER	WEIGHT	AMOUNT MIXTURE	RESULTS
		cc.	
5287	305	0.1	Dead 42 hours
5289	300	0.5	Dead 40 hours
5288	295	1.0	Dead 40 hours

All pigs developed typical toxic symptoms of tetanus with a slight localized area of infection. They died, unquestionably, from the free toxin present.

We next washed a culture twice and suspended the residue in 10 cc. physiologic salt solution. One-tenth cubic centimeter was injected intramuscularly. After 24 hours there was localized paralysis and at 82 hours complete paralysis, the pig dying after 96 hours. Again the reaction appeared to be due to free toxin.

In the next experiment the culture of *Cl. tetani* was washed twice, the residue taken up in 10 cc. physiologic salt solution and then heated in a water bath at 56°C. for 3 hours. One-tenth cubic centimeter was combined with 0.9 cc. of 10 per cent calcium chloride and 2 pigs injected intramuscularly, each with 0.1 cc.

Both pigs showed localized symptoms with paralysis of the hind-leg that had been injected, but the symptoms did not spread, and both pigs recovered.

We next increased the amounts of culture and calcium by combining 1 cc. of the washed and heated culture with 1 cc. of a 50 per cent solution of calcium chloride. Two guinea pigs were injected, one with 0.5 cc. and the other with 1 cc. Both de-

TABLE 1

Guinea pigs inoculated with C. tetani

DATE	GUINEA PIG NUMBER	WEIGHT	AMOUNT	RESULTS
7/24/35	To 1 cc. semi-solid agar culture (not washed or heated) added 9 cc. of 10 per cent $CaCl_2$. Injected the following amount:			
	5287	305 gm.	0.1 cc.	Dead 42 hours
	5289	300 gm.	0.5 cc.	Dead 40 hours
	5288	295 gm.	1.0 cc.	Dead 40 hours. Free toxin present
8/ 8/35	Culture washed twice and taken up in 10 cc. NaCl. Injected intramuscularly			
	4684	277 gm.	0.1 cc.	Dead 96 hours with a typical tetanus toxemia Free toxin still present
8/12/35	Washed culture heated at 56°C. for 3 hours. 0.1 cc. taken up in 0.9 cc. of 10 per cent solution $CaCl_2$			
	2663	460 gm.	0.1 cc.	Guinea pig 2663 had received 0.25 cc. alum toxoid one month previously. Guinea pig 5943 was normal. Neither pig developed tetanus
	5943	460 gm.	0.1 cc.	
9/ 5/35	To 1 cc. tetanus culture (washed and heated) added 1 cc. of 50 per cent $CaCl_2$			
	9313	415 gm.	0.5 cc.	Dead 100 hours — Normal pigs. Both pigs died with typical tetanus
	9034	410 gm.	1.0 cc.	Dead 88 hours
9/ 9/35	Used tetanus culture as prepared 9/5 for the balance of the experiments			
	2663	Treated	0.5 cc.	Localized tetanus. No general symptoms. Recovered
	5943	Control	0.5 cc.	Dead 90 hours, typical tetanus
9/13/35	9336	Treated	0.5 cc.	Localized tetanus
	9337	Treated	0.5 cc.	Localized tetanus
	9339	Treated	0.5 cc.	Localized tetanus
	9033	Control	0.5 cc.	Dead 88 hours, typical tetanus
9/23/35	2650	Treated	0.5 cc.	Localized tetanus
	2655	Treated	0.5 cc.	Localized tetanus
	2661	Treated	0.5 cc.	Localized tetanus
	9338	Treated	0.5 cc.	Localized tetanus
	9380	Control	0.5 cc.	Dead 112 hours, typical tetanus

veloped a severe localized infection with paralysis; on the third day they showed a generalized paralysis. The pig receiving 1 cc. died at 88 hours and the one receiving 0.5 cc. at 100 hours. This appeared to be an ideal mixture and in all other experiments it was used in a 0.5 cc. dose. This will be known as T. C. M. (tetanus spores and calcium chloride mixture).

TABLE 2

Potency of guinea pigs treated with tetanus alum precipitated toxoid

EXPERIMENT	PIG NUMBER	WEIGHT	0.25 CC. ALUM TOXOID	BLED 8/12/35	0.25 CC. ALUM TOXOID	BLED 8/19/35	BLED 8/26/35	0.25 CC. ALUM TOXOID	BLED 9/9/35	LIVE CULTURE	BLED 9/16/35	BLED 9/20/35	BLED 9/23/35	BLED 9/25/35	BLED 9/27/35	BLED 9/30/35
			1935	units	*1935*	units	units	*1935*	units	*1935*	units	units	units	units	units	units
I	2663	315	7/15	4		2	4			9/9	4				4	
II	9336	290	7/15	4	8/12		16			9/13		4			8	
	9339	290													4	
III	2650	315	7/15	4	8/12		16			9/23			4	−2	−2	2
	2655	315														
IV	9337	300	7/15	4			4	8/26		9/13		8			8	
V	2661	295	7/15	4			4	8/26	16	9/23			12	12	12	8
	9338	290														

EXPERIMENT I. INJECTION OF THE SPORES INTO IMMUNIZED GUINEA PIGS

On 7/15/1935, guinea pig 2663 was given 0.25 cc. of tetanus alum-precipitated toxoid subcutaneously. On 8/12/1935 (4 weeks later) its serum tested 4 units antitoxin per cubic centimeter. On 8/26/1935 (6 weeks later) it again tested 4 units per cubic centimeter. On 9/9/1935 this pig was inoculated intramuscularly into the right hind-leg with 0.5 cc. of T. C. M. Normal guinea pig 5943 was injected with the same volume of T. C. M.

Results. Guinea pig 2663 developed a localized infection in 72 hours with some local paralysis which gradually disappeared,

and the infection cleared up at the end of 2 weeks. The pig was bled 7 days after being infected and the serum still showed 4 units. It was again bled after 18 days and the titer remained 4 units. The control pig, 5943, developed a localized infection with paralysis in 48 hours which became generalized in 72 hours and the pig died at 90 hours with typical tetanus.

EXPERIMENT II

On 7/15/1935, 2 guinea pigs, each weighing 290 grams, were injected subcutaneously with 0.25 cc. tetanus alum precipitated toxoid. Four weeks later they were bled, and their combined sera showed a potency of 4 units per cubic centimeter.

Immediately after bleeding, they received a second injection of 0.25 cc. tetanus toxoid. They were bled 2 weeks after the second injection and their combined sera had a potency of 16 units per cubic centimeter.

On 9/13/1935, both pigs, together with a normal pig, received 0.5 cc. of T. C. M. The control died in 88 hours with typical tetanus.

One week later the treated pigs were bled and tested separately. Each serum had a potency of 4 units per cubic centimeter. A week later they were again bled and tested. One pig had a titer of 8 units antitoxin per cubic centimeter and the other of 4 units.

EXPERIMENT III

In experiment II the potency was much lower when tested after injecting the spores. Not knowing whether the potency had dropped before or after the spores were administered, we decided to bleed just before giving the spores in this experiment.

On 7/15/1935, two guinea pigs, each weighing 315 grams, were injected subcutaneously with 0.25 cc. tetanus alum-precipitated toxoid. Bleedings 4 weeks later tested 4 units. The pigs received a second injection of 0.25 cc. toxoid. Two weeks later their sera tested 16 units, but after 4 weeks the potency had dropped to 4 units.

Then the 2 pigs together with a normal pig were injected

intramuscularly with 0.5 cc. of T. C. M. The normal pig developed typical tetanus and died in 112 hours. The treated pigs had only a localized infection with complete recovery. They were bled 2 days after receiving the spores and tested −2 units per cubic centimeter; after 2 more days they tested −2 units and after 3 more days just 2 units.

A month after the second injection of toxoid, these pigs had dropped quite a bit in potency. After giving the spores there was another temporary drop, probably owing to a large amount of the available antitoxin being used to neutralize the massive infection, but within a week they were returning to the potency which was present before the infection.

EXPERIMENT IV

One guinea pig inoculated on 7/15/1935 with 0.25 cc. tetanus alum-precipitated toxoid when bled 4 weeks later tested 4 units, and at 6 weeks still tested 4 units. It was again injected with 0.25 cc. toxoid. Eighteen days later, this pig and a normal one were injected intramuscularly with 0.5 cc. T. C. M. The control pig developed typical tetanus and died in 88 hours. One week later the treated pig tested 8 units and after another week it still tested 8 units.

EXPERIMENT V

Two other pigs inoculated on 7/15/1935 with 0.25 cc. tetanus alum-precipitated toxoid showed 4 units of antitoxin 4 weeks later and the same after 6 weeks. On this date they were again inoculated with 0.25 cc. toxoid. Two weeks after their combined sera contained 16 units antitoxin per cubic centimeter, but after another 2 weeks they had dropped to 12 units.

On this date both pigs together with a normal pig were injected intramuscularly with 0.5 cc. T. C. M. The normal pig developed typical tetanus and died at 112 hours. The treated pigs were bled 48 hours after receiving the culture and their combined sera still tested 12 units. After 96 hours they were still 12 units, but after 7 days had dropped to 8 units.

DISCUSSION

The massive infective dose used in these tests may be considered unfair, but we wanted to obtain the extreme value. It is our intention in a further study to attempt to standardize an infective dose that will prove fatal in from 10 to 12 days.

The protocols would indicate that with the present infective dose, which proves fatal to a guinea pig in from 88 to 112 hours, it requires from 2 to 3 units of antitoxin per cubic centimeter of blood in the immunized pig to neutralize the toxin produced from the infection.

None of the treated pigs receiving the infective dose showed any marked increase in antitoxin within 18 days, but we shall continue to bleed these pigs at intervals and test their serum. It is possible that a smaller infective dose would permit an acceleration in antitoxin production, while the huge infective dose produced such a rapid infection that no time was allowed for acceleration in antitoxin. Also, our tests were made for 2, 4, 8, 12, and 16 units which are at very wide intervals, being as much as 50 to 100 per cent.

In testing human subjects after administering diphtheria toxoid, only enough antitoxin is required to neutralize a Schick test dose, which is 1/50th M.L.D. Contrasted with this one must consider that 1000 M.L.D. of tetanus toxin are required to neutralize 1 unit of antitoxin, so we may be expecting too much when we test for a titer of such a wide range as from 2 to 4 units of antitoxin.

These experiments would indicate the advisability of administering another dose of tetanus toxoid at the time of injury.

CONCLUSIONS

Guinea pigs that have received one or more injections of tetanus alum-precipitated toxoid, and are infected within 2 months, are protected against a massive dose of tetanus spores which kills normal pigs in from 88 to 112 hours.

A massive dose of tetanus spores does not markedly accelerate the production of antitoxin in guinea pigs that have been previously immunized with tetanus alum-precipitated toxoid.

REFERENCES

FILDES, P. 1927 Tetanus. VI. The conditions under which tetanus spores germinate in vivo. Brit. Jour. Exper. Path., **8**, 387.

FILDES, P. 1929a Tetanus. VIII. The positive limit of oxidation-reduction potential required for the germination of spores of *B. tetani* in vitro. Brit. Jour. Exper. Path., **10**, 151.

FILDES, P. 1929b Tetanus. IX. The oxidation-reduction potential of the subcutaneous tissue fluid of the guinea pig; its effect on infection. Brit. Jour. Exper. Path., **10**, 197.

GYE, W. E., AND CRAMER, W. 1919 Sixth Sc. Rep. Imp. Cancer Res. Fund, **40**, 57.

JONES, F. G. 1936 Studies on tetanus toxoid, II. In press.

JONES, F. G., AND MOSS, J. M. 1936 Studies on tetanus toxoid, I. Jour. Immunol., **30**, 115.

STUDIES OF STREPTOCOCCI

IV. RESISTANCE OF ENTEROCOCCI[1]

GEORGE H. CHAPMAN

Clinical Research Laboratory, 604 Fifth Avenue, New York, N. Y.

Received for publication February 11, 1936

In view of the anomalous results with enterococci mentioned in the first article of this series, special studies were made of these organisms. A summary of them will be presented in this paper.

Earlier American workers referred to the "enterococcus" as "*Streptococcus fecalis.*" However, English and German bacteriologists and recent American authors have referred to it as the "enterococcus." In the French literature it is known as "*Micrococcus ovalis,*" a term which was also adopted by Kendall and Haner (1924) in this country. The "*Streptococcus lactis*" of dairy workers is closely related. To avoid confusion with other fecal streptococci, and also because the organism has many properties distinct from other streptococci, the term "enterococcus" will be used in this paper.

Its habitat is mainly the intestinal tract. Of 909 strains studied in this laboratory, 80 per cent were recovered from human feces, while only 18.5 per cent of other streptococci were isolated from this source (table 1).

The outstanding characteristics of the organism are its extreme resistance, such as survival after exposure to 55°C. for 1 hour, and ability to grow in 20 per cent bile in peptone water or in extremely acid or alkaline media (pH 4 to 11). The enterococcus also possesses certain characteristic fermentative properties (aesculin, mannitol, etc.).

It grows well on common laboratory media, particularly those used for the isolation or differentiation of gram-negative intes-

[1] Aided by grants from the Ophthalmological Foundation, Inc.

tinal bacilli, e.g. Endo (Went, 1926, Yoshihara, 1933) and MacConkey (Anon, 1921, Downie and Cruikshank, 1928, and Torrey and Montu, 1936). To these should be added its ability to grow on Levine's eosine methylene-blue medium, which, because of the correlation with other properties and, because the medium was being used for other purposes, was employed for the differentiation of strains used in the present investigation. Most strains grew as well on Levine's medium as they did on nutrient agar or lactose agar.

In liquid media, growth occurs in short chains of cocci, but the stained appearance of growths from solid media more closely resembles that of the pneumococcus, except for the absence of a

TABLE 1

Frequency distribution of streptococci and enterococci

	EARLIER SERIES, STREPTOCOCCI	LATER SERIES	
		Streptococci	Enterococci
Total strains examined	4,561	3,477	909
Isolated from throat cultures	68%	63%	8%
Isolated from feces	18.5%	22%	80%

In the later series: 2,179 streptococci and 75 enterococci were isolated from the throat cultures; 753 streptococci and 730 enterococci were isolated from feces.

capsule. The pleomorphism of the organism has been commented on by almost every writer on the subject.

Considerable differences of opinion have existed as to whether the enterococcus is capable of producing changes in blood media similar to those produced by streptococci. However, the trend of recent thought is toward considering that similar changes occur in both organisms. All types have been isolated in this laboratory. Except for the slightly larger size of most enterococcus colonies and their greater compactness, the appearance of each blood-altering type was similar, regardless of whether the strain was an enterococcus or a streptococcus. It is, therefore, convenient to consider these types as "*Enterococcus, alpha*," "*Streptococcus, alpha*" etc.

RESISTANCE TO CHEMICALS, ETC.

It was mentioned previously that enterococci gave anomalous reactions to certain tests which were being applied to streptococci. The tests used were: (1) exposure to the "bactericidal" action of fresh, diluted, defibrinated guinea pig blood (Rawls and Chapman, 1935, and Chapman and Rawls, 1936); (2) exposure to the action of sodium bicarbonate, sodium carbonate and hexylresorcinol (Chapman and Rawls, 1936); and (3) intravenous injection of 5.0 cc. of live brain-heart infusion cultures into rabbits.

Enterococci, regardless of the blood-altering type, usually were resistant to the injurious agents used in tests (1) and (2). For example, when 75 strains were exposed to fresh, diluted, defibrinated guinea pig blood for 48 hours, and to 0.3 per cent sodium bicarbonate for 1 hour, 94.7 per cent were completely resistant to both reagents. They were resistant also when similarly exposed to 1:500,000 "Merthiolate," 1:2,500 phenol, 1:5,000 basic fuchsin, 1:50,000 hexylresorcinol and 1.0 per cent sodium carbonate. On the other hand, only 1 out of 16 streptococci was resistant to the latter group of reagents.

RESISTANCE AS COMPARED WITH OTHER ORGANISMS

In view of the extreme resistance of the enterococcus to these different reagents, its resistance was compared with that of *Escherichia coli*, *Aerobacter aerogenes*, staphylococci and other streptococci. Each strain was exposed to concentrations of sodium bicarbonate, sodium carbonate and sodium chloride varying from 0.1 to 5.0 per cent. Immediately on mixing, and again after 1 hour's incubation, transplants were made to suitable solid media. The growths on solid media were compared, and the degrees of reduction of growth after incubation were recorded from ++++ (no reduction) to 0 (complete reduction) (fig. 1).

As indicated from previous studies, streptococci were sensitive to certain dilutions of all three chemicals. However, enterococci were resistant to all three chemicals, even in the strongest concentrations used. *E. coli*, *A. aerogenes* and staphylococci resisted all concentrations of sodium bicarbonate and sodium chloride

but were sensitive to certain concentrations of sodium carbonate. Enterococci, therefore, were the most resistant of the organisms studied.

From these observations, it is clear that the bicarbonate and hexylresorcinol reactions proposed by Chapman and Rawls (1936) and Chapman and Curcio (1936) for the study of streptococci may not be appropriate for the study of the enterococcus group. This suggests the importance of differentiating enterococci (and, possibly, *S. lactis*) from other streptococci.

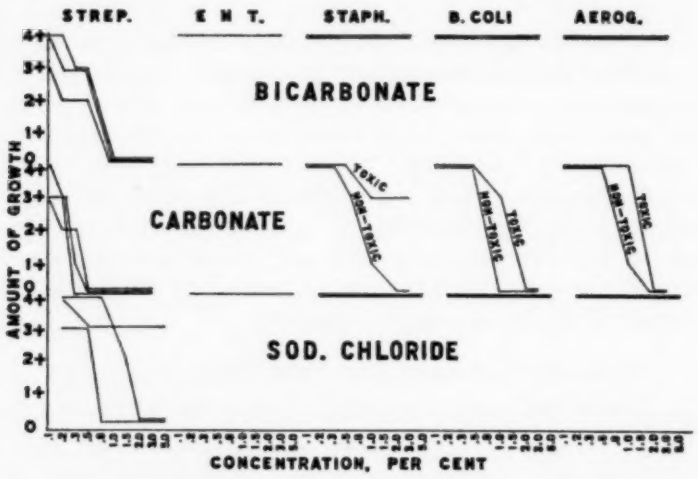


FIG. 1

LETHAL EFFECTS

Reports of the pathogenicity of the enterococcus have shown considerable variation. The organism has been isolated in pure culture from certain pathologic sources and, on this basis, has been considered pathogenic. However, animal inoculation experiments have given varying results.

Enterococci that were resistant to "bactericidin" (and, by inference, also resistant to chemicals) were injected intravenously into rabbits using 5.0 cc. of live cultures in brain heart infusion. Lethal effects were produced by only 5 of 20 strains and, in 3

of these, duplicate experiments with other rabbits failed to produce lethal effects (table 2). On the basis of this small series of tests, it would seem that most strains were not pathogenic for rabbits under the conditions of the experiments. In previously reported experiments (Chapman, Berens and Nilson, 1936)

TABLE 2

Effect of intravenous injection of 5.0 cc. of live brain heart infusion cultures of enterococci into rabbits (1,500 ± 100 grams)

STRAIN NUMBER	TYPE	"BACTERICIDIN" REACTION	EFFECT ON RABBITS
3417	Alpha	++++	Died overnight
3585	Alpha	++++	Died overnight
3418	Alpha	++++	Survived*
3445	Alpha	++++	Survived
3432	Alpha	+++	Survived
3480	Alpha	++++	Survived
3436	Beta	++++	Survived
3456	Beta	++++	Survived
3519	Beta	++++	Survived
3507	Gamma	++++	Survived
6167	Alpha	++++	Died, 2 days Survived
6240	Alpha	++++	Survived Survived
6297	Alpha	++++	Survived Died, overnight
6479	Alpha	++++	Died, 4 days Survived
6570	Alpha	++++	Survived Survived

* Survived 10 days.

streptococci that were resistant to "bactericidin" usually produced lethal effects under similar conditions.

Insufficient data are available on experiments with killed ("Merthiolate") cultures.

SUMMARY

The enterococcus (syn. *Streptococcus fecalis, Micrococcus ovalis* and, possibly, *Streptococcus lactis*) was resistant to a number of

injurious agents. Of 75 strains tested, 70 resisted the action of 1:500,000 "Merthiolate," 1:2,500 phenol, 1:5,000 basic fuchsin, 1:50,000 hexylresorcinol and 1.0 per cent sodium carbonate for 1 hour. In contrast, only 1 out of 16 smooth streptococci was resistant to these agents.

When compared with *Escherichia coli*, *Aerobacter aerogenes*, staphylococci and streptococci, enterococci were the most resistant to sodium carbonate, sodium bicarbonate and sodium chloride. In fact, enterococci were still viable after contact for 1 hour with 3.0 per cent sodium carbonate.

Enterococci usually did not kill rabbits when 5.0 cc. of their live brain-heart infusion cultures were injected intravenously. In previous experiments, smooth streptococci that were resistant to injurious agents usually killed rabbits under similar conditions.

It is suggested that interpretation of resistance to injurious agents will be simplified if enterococci are differentiated from other streptococci.

The authors wish to express their appreciation to Drs. Merritt H. Stiles and Clarence W. Lieb for their help in this series of studies and in the preparation of the manuscripts.

REFERENCES

ANON 1921 Med. Res. Council, Special Reports Series, No. 51, p. 14.
BAGGER, S. V. 1924 Jour. Path. and Bact., **29**, 225.
CHAPMAN, G. H., BERENS, C., AND NILSON, E. 1936 Jour. Bact., **31**, 339.
CHAPMAN, G. H., AND CUBCIO, L. 1936 Jour. Bact., **31**, 333.
CHAPMAN, G. H., AND RAWLS, W. B. 1936 Jour. Bact., **31**, 323.
DOWNIE, A. W., AND CRUIKSHANK, J. 1928 Brit. Jour. Exper. Path., **9**, 171.
KENDALL, A. I., AND HANER, R. C. 1924 Jour. Infect. Dis., **35**, 67.
RAWLS, W. B., AND CHAPMAN, G. H. 1935 Jour. Lab. and Clin. Med., **21**, 49.
TORREY, J. C., AND MONTU, E. 1936 Jour. Infect. Dis., **58**, 105.
WENT, S. 1926 Centralbl. f. Bakt., 1 Abt., Orig., **100**, 62.
YOSHIHARA, R. 1933 Centralbl. f. Bakt., 1 Abt., Orig., **128**, 462.

THE BACTERICIDAL AND PHOTOCHEMICAL PROPERTIES OF IRRADIATED COD LIVER OIL AND AN OZONIDE OF OLIVE OIL

FRANKLIN A. STEVENS

Department of Medicine, College of Physicians and Surgeons, Columbia University and the Presbyterian Hospital, New York City

Received for publication February 20, 1936

The bactericidal and photochemical properties of oils exposed to ultraviolet light have been studied by many investigators. Both these effects were, at first, attributed to invisible light rays but recent experimentation has shown that bacteria are killed and photographic films are fogged by vapor from the oils (references in bibliography). Following the observation of Proctor and Milas (1931) that germicidal peroxides were formed by the exposure of certain substances to ultraviolet light, Harris, Bunker and Milas (1932) studied the peroxide content and the bactericidal effect of vapors from various irradiated oils. They concluded that the germicidal effect of the emanations from these irradiated oils was due to substances of a gaseous nature composed partly of volatile peroxides. Recent studies show that bactericidal peroxides occur in irradiated cod liver oil and in some irradiated essential oils (Stevens, 1936 a and b). In the following experiments the bactericidal properties of irradiated cod liver oil have been further investigated, the germicidal effects of an ozonide of olive oil have been studied and the bactericidal and photochemical properties of the vapors from these oils have been correlated.

BACTERICIDAL EMULSIONS OF ULTRAVIOLET-IRRADIATED COD LIVER OIL

In previous experiments it was found that peroxides were the only bactericidal substances in emulsions of irradiated cod liver

oil (Stevens 1936 a). These peroxides adhered to bacteria suspended in the emulsions and their bactericidal action was continued after the bacteria were removed from the emulsions and resuspended in dilute reducing solutions. No attempt was made to study the effect on a variety of bacteria or to determine the optimum period of irradiation. In the following experiments the bactericidal properties of emulsions of cod liver oil irradiated for 3, 6, 9, 12 and 15 hours have been determined. The germicidal effect of the emulsions on the common upper respiratory bacteria has been investigated. Emulsions made with salt solution have also been compared with emulsions made with a buffered phosphate mixture. This comparison was necessary because the emulsions made in salt solution were quite acid.

Experiments I and II. The bactericidal properties of emulsions of cod liver oil irradiated with ultraviolet light

A commercial sample of cod liver oil was distributed among several large Petri dishes to a depth of 0.5 cm. These dishes were placed under a mercury-quartz-arc at a distance of 20 inches. At the end of 3, 6, 9, 12 and 15 hours two samples of 4 cc. each were removed and placed in sterile bottles. To one of these samples, 16 cc. of sterile salt solution were added. To the second were added 16 cc. of sterile buffered phosphate mixture (KH_2PO_4, Na_2HPO_4, M/15, pH 7.4). The bottles were covered with rubber caps, shaken one hour in a machine, then inverted and allowed to stand overnight in the cold to permit the unemulsified oil to separate.

Three strains of hemolytic streptococcus (273, C 203, and NY V) and two strains of *Staphylococcus aureus* (S and E) were grown for 18 hours in proteose peptone broth. Nine cubic centimeters of each of these cultures were used to test the bactericidal effect of oil irradiated for 3, 6, 9, 12 and 15 hours and emulsified either in salt solution or in phosphate mixture. Nine cubic centimeters of each culture were centrifuged and the bacterial sediment was suspended in 3 cc. of the respective emulsions aspirated through the rubber cap without disturbing the supernatant layer of oil. The suspensions were cultured at hourly intervals on blood agar plates. The control cultures in emulsions of normal oil have been

TABLE 1

The bactericidal properties of emulsions of irradiated cod liver oil

IRRADIATION	STRAIN				
	273 (H. S.)	N. Y. V. (H. S.)	C 203 (H. S.)	Staphylococcus S	Staphylococcus E
Emulsified in salt solution					
hours					
3	D 22 hr.	D 22 hr.	D 22 hr.	L 72 hr.	L 72 hr.
6	S 6 D 22	D 22	S 6 D 22	L 72	S 48 D 56
9	S 5 D 6	S 5 D 6	S 5 D 6	D 30	S 22 D 30
12	S 5 D 22	S 4 D 5	S 4 D 5	D 30	
15	D 4	D 4	D 4		
Emulsified in phosphate solution, M/15, pH 7.4					
3	D 22	D 22	D 22	L 46	L 46
6	S 6 D 8	S 8 D 22	S 8 D 22	D 22	D 30
9	S 6 D 7	S 6 D 7	S 5 D 8	D 22	D 22
12	S 5 D 7	S 5 D 6	S 5 D 6	D 22	S 5 D 8

TABLE 2

The bactericidal effects of emulsions of cod liver oil on upper respiratory tract bacteria

	STRAIN							
	Pneumococcus I	Pneumococcus II	Pneumococcus III	Pneumococcus bacillus	*Streptococcus viridans*	*Staphylococcus aureus*	*Micrococcus catarrhalis*	Hemolytic streptococci
Normal oil emulsified in phosphate mixture	D 30 hr.	D 30 hr.	S 22 hr. D 30 hr.	L 78 hr.	L 30 hr.	L 78 hr.	D 60 hr.	L 48 hr.
Irradiated oil emulsified in salt solution	D 1	D 1	S 1 D 2	S 1 D 2	S 2 D 3	D 4	D 2	S 5 D 8
Irradiated oil emulsified in phosphate mixture	S 2 D 3	D 3	D 3	D 2	S 4 D 5	S 7 D 8	D 4	S 5 D 7

omitted. These were all living at the end of 48 hours. The results of these cultures are to be found in table 1. In this table L indicates an abundant growth, S scant growth, and D no growth.

In experiment II, the bactericidal properties of emulsions of oil irradiated for 12 hours were studied. The common upper respiratory pathogenic bacteria were used in these experiments (table 2).

THE BACTERICIDAL PROPERTIES OF AN OZONIDE OF OLIVE OIL

While irradiation renders most fish oils bactericidal by increasing the peroxide content of the oils, most vegetable oils are

TABLE 3

The bactericidal properties of an ozonide of olive oil

	BACTERIUM					
	Pneumococcus	Friedlander bacillus	Hemolytic streptococci	*Micrococcus catarrhalis*	*Streptococcus viridans*	*Staphylococcus aureus*
Number of bacteria	25[9]	50[9]	80[9]	50[9]	80[9]	110[9]
Salt solution emulsion O = 0.005 gram per 100	S 3 hr. D 4	D 18 hr.	D 18 hr.	S 4 hr. D 8	D 4 hr.	L 30 hr.*
Ozonide						
O = 0.03 gram per 100	D 3 hr.	L 7 hr.*	D 4 hr.	D 3 hr.	L 7 hr.*	L 24 hr.*
O = 0.06 gram per 100	D 2 hr.	D 7 hr.	D 3 hr.	D 10 min.	D 4 hr.	D 5 hr.
O = 0.13 gram per 100	D 2 hr.	D 4 hr.	D 1 hr.	D 4 min.	D 30 min.	D 5 hr.
O = 0.26 gram per 100	D 20 min.	D 1 min.	D 1 min.	D 1 min.	D 15 min.	D 4½ hr.
O = 0.52 gram per 100	D 2 min.	D 1 min.	D 1 min.	D 1 min.	D 3 min.	D 3½ hr.
Emulsion in serum	D 6 hr.	S 6 hr. D 20 hr.	D 6 hr.	D 6 hr.	S 7 hr. D 20 hr.	D 40 hr.

* Culture discontinued.

not so affected by this treatment. Irradiation has little or no effect on olive oil but by ozonization of the oil oxygen can be added to the double bond. Harada (1934) prepared ozonides of olive oil and found them fungicidal. In the following experiment the bactericidal effects of an ozonide of olive oil have been investigated (experiment III). The effect of suspending bacteria in emulsions of ozonide in salt solution and in diluted serum have been studied. The bacteria were also suspended in the ozonide diluted in mineral oil so that it contained varying percentages of available oxygen.

Experiment III. The bactericidal properties of olive oil ozonide

A sample of ozonide of olive oil 24 months old was used in these experiments. This oil originally contained 1.3 grams per cent of available oxygen, of which it had lost approximately 30 per cent. A few cubic centimeters of this oil were diluted with mineral oil so that the mixture contained 0.3 gram per cent of oxygen. One part of the mixture was shaken for one hour in a shaking machine with isotonic phosphate mixture (pH 7.4). The rubber-capped flask containing the emulsion was then inverted in the cold overnight. Cultures of pneumococcus, Friedlander bacillus, hemolytic streptococcus, *Micrococcus catarrhalis*, *Streptococcus viridans* and *Staphylococcus aureus* were grown eighteen hours. Six cubic centimeters of these cultures were centrifuged and the sediment was suspended in 2 cc. of the emulsion withdrawn through the cap of the inverted flask. Cultures of these suspensions were made at frequent intervals on blood agar plates.

In the second part of the experiment the sediment from 6 cc. of culture was suspended in 2 cc. of mixtures of ozonide of olive oil with mineral oil containing varying percentages of available oxygen. At intervals these suspensions were cultured in broth. After incubation these broth cultures were inoculated on plates.

In the third part of the experiment ozonide diluted to contain 0.5 gram per cent of available oxygen was shaken with rabbit serum, diluted twice with salt solution. The bactericidal effect of the resulting emulsion was studied.

THE CORRELATION OF THE FOGGING AND BACTERICIDAL EFFECTS OF THE VAPOR FROM IRRADIATED COD LIVER OIL AND AN OZONIDE OF OLIVE OIL

The emanation from irradiated oils which fogs photographic plates is evidently of a gaseous nature because, while it can penetrate films of aceto-cellulose (Schmidt, 1908), glass, metal or quartz interposed between the oil and the plates abolish the effect. The vapor arising from irradiated oils contains both peroxides and aldehydes (Stevens, 1935). In previous experiments the peroxides in irradiated oils were shown to be the only bactericidal substances formed as a result of irradiation. Chemi-

cals which destroyed the peroxides rendered the irradiated oils non-bactericidal. In the following experiment irradiated oils and ozonides of olive oil have been treated with both organic and inorganic chemicals to reduce the peroxides and ozonides. The vapor from these irradiated oils no longer fogged plates.

Experiment IV. The photochemical effects of vapor from irradiated cod liver oil, mineral oil, and an ozonide of olive oil

Chemicals destructive to peroxides were added directly to the oils in this experiment and the fogging effect of the vapor from the treated oils was studied. Normal mineral oil which did not fog plates was used as a diluent and as a vehicle to test the fogging effect of the chemicals used. Five sets of tests were done. The first was a control set of tests in mineral oil. To each of three 5 cc. lots of normal oil were added 1 gram of cysteine, cystine, and alanine respectively. One-half gram of powdered KI and a few drops of glacial acetic acid were added to a fourth sample. The KI and acetic acid added to a fifth tube were neutralized by adding 0.5 gram of $CaCO_3$ a few hours previous to testing the vapor; 0.5 gram of I was added to a sixth tube. After a few days 1 cc. samples of each of these treated oils were placed in small dishes with ground glass lips and photographic plates were inverted over the dishes. The plates were developed after 24 hours. In a second series of tests the chemicals mentioned above were mixed with cod liver oil which had been irradiated for 12 hours. These mixtures were shaken frequently for a week. In a third series the cod liver oil was allowed to stand in contact with the chemicals for two weeks before the photochemical qualities of its vapor were tested. A fourth series of tests was done with ozonide of olive oil diluted with mineral oil so as to contain 0.1 gram per 100 of available oxygen. The ozonide was allowed to stand for one week before testing. In a fifth series irradiated mineral oil was used. The effect of adding these chemicals to the oils has been charted in table 4 in which the degrees of blackening caused by the vapor from the treated oils have been indicated by + signs.

In the previous experiment (experiment IV) the fogging effect

of the vapor from irradiated mineral oil and cod liver oil was affected only by the addition of cysteine and of acetic acid and potassium iodide which abolished the effect after a few days' contact with the oils. While the potassium iodide and acetic acid destroyed the fogging properties of the vapor from the cod liver oil within a week, the oil required two weeks in contact with cysteine before photographic plates were not affected. The initial blackening effect of the cod liver oil was much more intense and the concentration of peroxides, much greater than in the mineral oil, probably accounting for the greater length of time necessary for the completion of the reducing effect of the cysteine. While neither cystine, alanine, nor iodine affected

TABLE 4

The photochemical effects of vapor from irradiated oils and ozonide of olive oil

VARIETY OF OIL	IRRADIATION	DURATION OF CONTACT	UNTREATED OIL	OIL AND CYSTEINE	OIL AND CYSTINE	OIL AND ALANINE	OIL, KI AND ACETIC ACID	OIL, KI, ACETIC AND $CaCO_3$	OIL AND IODINE
	hours	*days*							
Mineral oil		7	−	−	−	−	±	−	−
Mineral oil	8	7	+	−	+	+	±	−	+
Cod liver oil	12	7	++++	++	++++	++++	±	−	++++
Cod liver oil	12	14	++++	−	++++	++++	±	−	++++
Ozonide of olive oil		7	++++	−	++	+++	±	−	+++

the plates or interfered with the photochemical action of the vapor from the mineral oil or cod liver oil, they definitely affected the ozonide, suggesting that it was a more active oxidizing agent than the irradiated oils. Various degrees of blackening of the plates were obtained by the addition of cysteine, cystine and alanine to the ozonide so that it has been possible to compare different degrees of fogging with the bactericidal effect on *Staphylococcus aureus*. One cubic centimeter samples of the ozonide which had been treated with these amino acids were placed in dishes similar to those used in testing the effect of the vapor on photographic plates. One cubic centimeter of nutrient agar was poured into a second set. This agar when cooled was sprayed

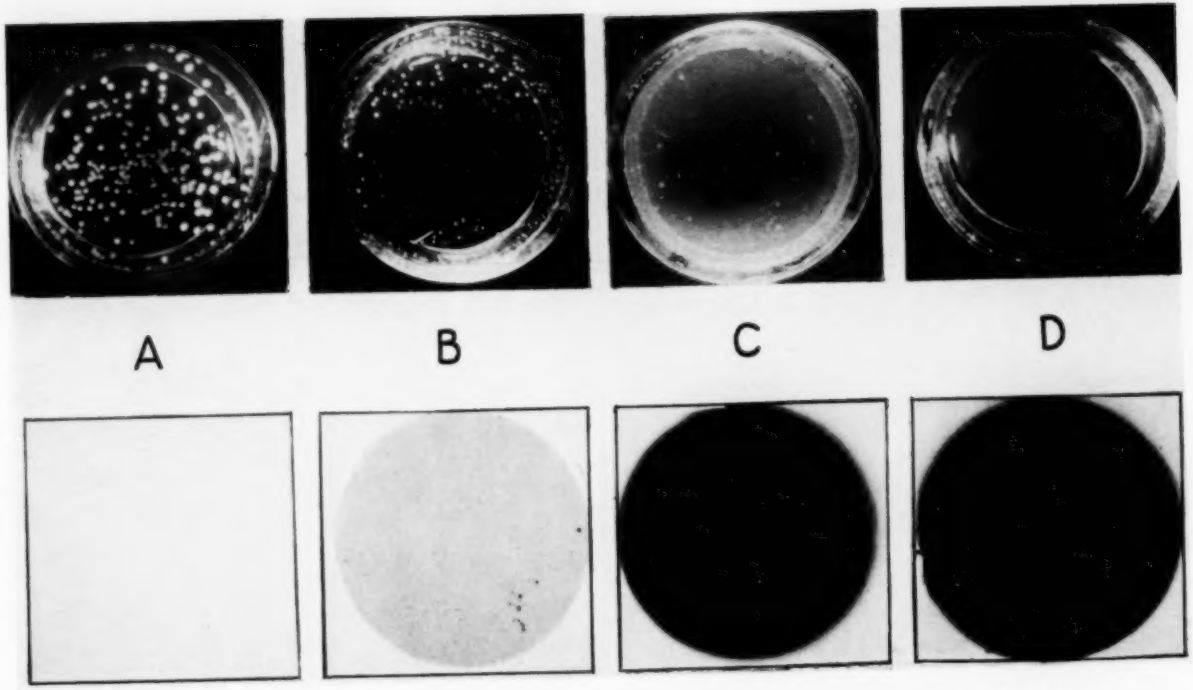


FIG. 1. COMPARISON OF FOGGING AND BACTERICIDAL EFFECTS OF VAPOR FROM OZONIDES OF OLIVE OIL

A, in contact with cysteine; *B*, in contact with cystine; *C*, in contact with alanine; *D*, ozonide in mineral oil; (0.1 gram per 100 available O).

with a culture of *Staphylococcus aureus* diluted so that the colonies would be discrete. These seeded agar dishes were inverted over the dishes of oil and the junction sealed with tape. After twenty-four hours' incubation the colonies developing on these dishes were photographed and compared with the degree of blackening obtained with similar samples of oil. The photographs of the seeded plates and the degrees of blackening obtained with the corresponding samples of oil have been reproduced in figure 1. A comparison of the fogging and bactericidal effects obtained in this experiment shows that they are quantitatively related. The rate of growth of the bacteria is also affected, since the colonies on the plates exposed to vapor from oils which fogged the plates more intensely are smaller. That the growth rate of bacteria might be affected by suspending them for a short time in emulsions of irradiated cod liver oil in salt solution was observed in some of the previous experiments. In experiments I and II where growth was scant the majority of the colonies were small. Permanent dissociation of a stable M pneumococcus or Friedlander bacillus to an intermediate MS variant has also occurred under these conditions.

SUMMARY AND CONCLUSIONS

In the experiments described the fogging and bactericidal effects of the vapor from irradiated cod liver oil and from ozonide of olive oil have been found to be due to substances liberating active oxygen. Ozonides of olive oil, emulsions of ozonide in salt solution and in serum, and the vapor from ozonides are all germicidal. Bacteria are not only killed by the oxygen liberated from these oils but their growth rate may be retarded or dissociation may occur if the bacteria are subjected to sublethal doses.

The ozonide of olive oil used in some of these experiments was obtained through the courtesy of the Johnson Laboratories, New York City.

REFERENCES

HARADA, T. 1934 Olive oil ozonide and its fungicidal quality. Chem. Soc. Jap. Bull., **9**, 192.

HARRIS, R. S., BUNKER, J. W. M., AND MILAS, N. A. 1932a The germicidal activity of vapors from irradiated oils. Jour. Bact., **3**, 429.

HARRIS, R. S., BUNKER, J. W. M., AND MILAS, N. A. 1932b The chemical nature of germicidal vapors emanating from irradiated oils. Ind. Chem. Eng., **24**, 1181.

KUGELMASS, I. N., AND MCQUARRIES. 1924 I. The photoactivity of substances curative of rickets and the photolysis of the oxy-products by ultraviolet irradiation. Science, **60**, 1551, 272.

MANSVILLE, I. A. 1925 Effects of radiations of cod liver oil. Jour. Amer. Med. Assoc., **84**, 1401.

PROCTOR, B. E., AND MILAS, M. A. 1931 The bactericidal efficiency of certain organic peroxides. Jour. Bact., **21**, 54.

SCHMIDT, W. 1908 Radiation from drying oils. Z. physik. Chem., **64**, 243.

SEARS, H. J., AND BLACK, N. 1934 The bactericidal properties of ultraviolet irradiated petrolatum. Jour. Bact., **27**, 453.

STEVENS, F. A. 1935 The bactericidal and photochemical properties of irradiated petrolatum and mineral oil. Jour. Lab. and Clin. Med. **21**, 26.

STEVENS, F. A. 1936a The bactericidal effect of peroxides in irradiated cod liver oil. Jour. Inf. Dis. (In press).

STEVENS, F. A. 1936b The bactericidal action of irradiated oil of pine on hemolytic streptococcus. Jour. Lab. and Clin. Med. (In press).

THOMPSON, L. AND SHEARD, C. 1931 The bactericidal action of irradiated oils. Proc. Staff Meetings, Mayo Clinic, **6**, 46, 698.

WRENN, H. T. 1927 Studies on the bactericidal properties in vitro of certain fatty oils irradiated with the quartz-mercury-vapor spectrum. U. S. Vet. Bur. Med. Bull., **3**, 898

EFFECTIVENESS OF HOT HYPOCHLORITES OF LOW ALKALINITY IN DESTROYING MYCOBACTERIUM TUBERCULOSIS

S. M. COSTIGAN

General Laboratories, Division of Pennsylvania Salt Manufacturing Company, Philadelphia, Pennsylvania

Received for publication February 21, 1936

The feeling expressed by some writers that sterilization by hypochlorites of milk plant apparatus, dairy equipment and restaurant dishes was not complete because of the inability of these products to destroy *Mycobacterium tuberculosis* stimulated a study to determine the effectiveness of hot hypochlorite solutions in killing this organism. Tilley (1920) found that Dakin's solution, 1:500 (10 parts of available chlorine per million) was ineffective in 10 minutes, while 1:200 dilution (25 parts of available chlorine per million) was effective in destroying *M. tuberculosis*.

Whittaker (1928) showed that the calcium hypochlorite in concentrations up to 500 parts of available chlorine per million for ½ minute used to rinse pint milk bottles contaminated with sputum from tuberculous patients was not effective in destroying *M. tuberculosis*. This work was done at room temperature.

Rice (1933) reported that a strongly alkaline hypochlorite, antiformin, 1 part of undiluted antiformin to 5 parts of sputum from tuberculous patients, destroyed this organism in 10 minutes.

We found that when hypochlorite solutions of low alkalinity containing 50, 100, and 200 parts available chlorine per million are heated to a temperature ranging between 54 to 58°C. for a period of three hours there is no loss in their available chlorine concentrations. Even boiling these solutions does not diminish their available chlorine.

Solutions of the hypochlorite containing 50 and 200 parts of

available chlorine per million and temperatures of 50°, 55° and 60°C. with exposures of ½, 1, 2½ and 5 minutes were chosen because at these temperatures and times, *M. tuberculosis* in water is not destroyed. In addition to this, concentrations of 50 to 200 parts available chlorine per million of water are used as bactericides for dairy and food equipment.

TECHNIC

The organism used for the first series of inoculations was a 2 months' old culture of *M. tuberculosis* human strain No. 4777, obtained from the American Type Culture Collection. As it was impossible to get an even suspension of later cultures of No. 4777, a culture (A 14) for the second run was obtained from the Henry Phipps Institute, Philadelphia, and one for the third run, from Saranac Laboratory, Saranac Lake, New York.

The growths were washed from the slants with sterile normal saline and placed in flasks containing glass beads. The suspension was shaken vigorously to break up the clumps, and then so diluted that 2 cc. added to the 20 cc. of disinfectant dilution would give a dosage of 20,000,000 organisms when 1 cc. of the final centrifuged mixture was injected into a guinea pig.

The hypochlorite was heated in a water bath to the desired temperature and held for a few minutes before the suspension of organisms was added. At intervals of ½, 1, 2½ and 5 minutes, 2 cc. of the suspension were withdrawn and 1 cc. was pipetted into each of two centrifuge tubes containing 5 cc. of sterile N/10 sodium thiosulphate to act as an antichlor. The suspension was centrifuged for 10 minutes and the supernatant removed, leaving 1 cc. in the tube which was injected into the left inguinal lymph gland region of a 250-gram guinea pig. Two animals, therefore, were injected for each time, temperature and concentration.

It was believed desirable to determine the effect of the culture upon the rate of deterioration of the available chlorine of the solution. Four hundred million organisms were added to 20 cc. of the disinfectant dilution when it had reached the desired temperature. These tests were run in a manner similar to those from which the guinea pig inoculations were made. The solutions were titrated before and after the tests were made.

Control tests were made under identical conditions except that water instead of hypochlorite solutions was used.

In later experiments, the procedure was the same except the organism suspension was filtered after shaking, and then counted.

The animals were autopsied eight to twelve weeks after injection, and smears for acid fast bacilli were made from all organs showing lesions. In one small group of animals which were

TABLE 1

Effectiveness of solutions containing 50 parts available chlorine per million parts of water in destroying Mycobacterium tuberculosis

TEMPERATURE	TIME	LEFT INGUINAL LYMPH GLAND	LUNGS	LIVER	SPLEEN	SITE OF INOCULATION
°C.	minutes					
50	½	+	±	±	±	±
	1	±	±	±	±	±
	2½	−	−	−	−	−
	5	−	−	−	−	−
55	½	±	−	−	±	±
	1	−	−	−	−	−
	2½	−	−	−	−	−
	5	−	−	−	−	−
60	½	−	−	−	−	−
	1	−	−	−	−	−
	2½	−	−	−	−	−
	5	−	−	−	−	−

Grouping of the results of 3 runs of the experiment.

+, lesions with acid-fast bacilli; ±, lesions with acid-fast bacilli in 1 run negative in 2 runs; −, no lesions.

tuberculin tested, the results of the tuberculin test supported the post mortem findings.

The hypochlorite used was B-K[1] which is essentially a solution of sodium hypochlorite containing 3.50 per cent, and is alkaline with calcium hydrate 0.12 per cent. At 20°C. using the Leeds and Northrup glass electrode the pH of the 50 p.p.m. solution was found to be 8.35, and that of the 200 p.p.m. solution 9.04.

[1] Manufactured by General Laboratories.

RESULTS

Table 1 shows the effectiveness of solutions containing 50 p.p.m. of available chlorine in destroying *M. tuberculosis*. At 50°C., this solution failed to kill in ½ minute. All animals showed tuberculosis with acid-fast bacilli in the left inguinal lymph glands. In one run the animals showed lesions and acid-fast bacilli in the lungs, liver and spleen with abscess formation at the site of inoculation. With 1-minute exposures, the animals in one

TABLE 2

Effectiveness of solutions containing 200 parts available chlorine per million parts of water in destroying Mycobacterium tuberculosis

TEMPERATURE	TIME	LEFT INGUINAL LYMPH GLAND	LUNGS	LIVER	SPLEEN	SITE OF INOCULATION
°C.	minutes					
50	½	±	−	−	−	−
	1	±*	−	−	−	−
	2½	−	−	−	−	−
	5	−	−	−	−	−
55	½	−	−	−	−	−
	1	−	−	−	−	−
	2½	−	−	−	−	−
	5	−	−	−	−	−
60	½	−	−	−	−	−
	1	−	−	−	−	−
	2½	−	−	−	−	−
	5	−	−	−	−	−

Grouping of the results of 3 runs of the experiment.

+, lesions with acid-fast bacilli; ±, lesions with acid-fast bacilli in 1 run negative in 2 runs; −, no lesions; *, lesions, no acid-fast bacilli.

run showed tuberculosis with lesions and acid-fast bacilli in the left inguinal lymph glands while all others were negative. Animals receiving the organisms subjected to hypochlorite solutions of 50 parts available chlorine at 50°C. in times of 2½ and 5 minutes did not show tuberculosis.

When the solution was heated to 55°C., exposures of ½ minute gave irregular results. In some runs characteristic lesions

developed with acid-fast bacilli in the inguinal lymph glands and spleen and at the site of inoculation, while in other runs the

TABLE 3

Water—control

TEMPERATURE	TIME	LEFT INGUINAL LYMTH GLAND	LUNGS	LIVER	SPLEEN	SITE OF INOCULATION
°C.	minutes					
50	½	+	±	±	+	+
	1	+	±	±	+	+
	2½	+	±	±	+	+
	5	+	±	±	+	+
55	½	+	±	±	±	+
	1	+	±	±	±	+
	2½	+	±	±	±	±
	5	+	±	±	±	±
60	½	+	±	±	±	±
	1	+	±	±	±	±
	2½	+	±	±	±	±
	5	+	±	±	±	±

Grouping of the results of 3 runs of the experiment.

+, lesions with acid-fast bacilli; ±, lesions with acid-fast bacilli in 1 run negative in 2 runs; −, no lesions.

TABLE 4

Deterioration of hypochlorite in 5 minutes after addition of organisms

TEMPERATURE		50 P.P.M.	200 P.P.M.
°C.			
50	Start	53.95	199.5
	End	47.96	190.0
55	Start	53.95	199.5
	End	47.10	187.5
60	Start	53.95	199.5
	End	45.35	186.0

inoculated animals did not develop tuberculosis. Animals receiving organisms which had been exposed for 1, 2½ and 5 minutes to the 50 parts available chlorine per million at 55°C.

were negative, indicating that the organisms had been killed. Likewise complete destruction even at ½ minute at 60°C. was obtained.

Table 2 shows the effectiveness of solutions containing 200 p.p.m. of available chlorine in destroying *M. tuberculosis*. This solution gave irregular results when heated to 50°C. for ½ minute.

In one run the animals receiving the organisms exposed for 1 minute developed lesions at the site of inoculation. No acid-fast bacilli could be demonstrated nor could the disease be reproduced by injection of the emulsified lesions into susceptible animals. Whether this is the same phenomenon observed by Sabin and Anderson (1932) after the injection of the phosphatide fraction of these organisms cannot be said. All the remaining 50°C. animals as well as all of the 55°C. and 60°C. were negative.

Table 3 gives the findings in the control animals. All showed lesions with acid-fast bacilli in the left inguinal lymph glands, the region of inoculation. All of the 50°C. animals showed lesions and acid-fast bacilli in the spleen as well as at the site of inoculation. In some runs, lesions with acid-fast bacilli were present in the lungs, liver and spleen in all animals while in some the lesions were absent. This was attributed to the differences of the virulence of the cultures used.

Table 4 shows the effect of the number of organisms used in this experiment upon the deterioration of the hypochlorite solutions during a period of 5 minutes with the temperatures employed.

CONCLUSIONS

Heating suspensions of *Mycobacterium tuberculosis* (human strain) to 60°C. for 5 minutes does not destroy the organism. The hypochlorite solution of low alkalinity, containing 50 parts available chlorine per million parts of water heated to 50°C., destroys *Mycobacterium tuberculosis* in 2½ minutes; heated to 55°C. it destroys it in 1 minute; and heated to 60°C., it destroys it in ½ minute.

The hypochlorite solution of low alkalinity containing 200 parts available chlorine per million parts of water heated to 50°C. destroys *Mycobacterium tuberculosis* in 1 minute; heated to 55°C. and 60°C., it destroys it in ½ minute.

The author wishes to acknowledge the kind assistance given by Messrs. J. W. Yates and W. A. Hadfield in this work.

REFERENCES

RICE, J. W. 1933 Tenth Annual Report, The Pennsylvania Association of Dairy and Milk Inspectors (1934).

SABIN, F. R., AND ANDERSON, R. J. 1932 Physiol. Rev., **12**, 141.

TILLEY, F. W. 1920 Jour. Agr. Res., **20**, 85–110.

TILLEY, F. W., MACDONALD, A. D., AND SCHAFFER, J. M. 1931 Jour. Agr. Res., **42**, 93–106.

WADE, E. M., ARCHBALD, R. W., AND WHITTAKER, H. W. 1928 Jour. Bact., **15**, 189–194.

HYDROGEN SULFIDE PRODUCTION AS A DIFFERENTIAL TEST IN THE COLON GROUP

REESE VAUGHN AND MAX LEVINE

Iowa Engineering Experiment Station, Iowa State College, Ames, Iowa

Received for publication February 24, 1936

I. HISTORICAL

Gayon (1877) was probably the first to demonstrate the formation of H_2S by bacterial action. He employed lead acetate as an indicator. Since that time the phenomenon of H_2S production by bacteria has received considerable attention.

The production of H_2S in a suitable medium has long been used as a means of differentiation of species in the colon-typhoid group of bacteria. Orlowski (1895) reported the blackening of lead acetate in a peptone medium by *Eberthella typhosa* but not by *Escherichia coli*. Sacquépée and Chevrel (1905) confirmed the work of Orlowski and noted further that paratyphoid B blackened lead acetate, iron potassium tartrate and nickel sulfate whereas paratyphoid A did not.

Burnet and Weissenbach (1915) confirmed the results of Sacquépée and Chevrel and advocated the use of lead acetate agar for differentiating paratyphoid A and B.

Jordan and Victorson (1917), Kligler (1917) and others used and recommended lead acetate agar media for detecting H_2S formation by colon-typhoid bacteria. Wilson (1923) employed a ferric chloride medium to detect sulfides as did Beckwith and Moser (1932).

Levine and co-workers (1932), in an attempt to develop a medium better suited for use with the colon-aerogenes group of bacteria than the lead acetate media previously referred to, employed nickel, iron, lead and manganese salts as indicators of H_2S in peptone media. They suggested an agar medium which contained 2.0 per cent proteose peptone (Difco) with 0.05 per cent ferric citrate as the indicator. This medium was particu-

HYDROGEN SULFIDE PRODUCTION AS A DIFFERENTIAL TEST IN THE COLON GROUP

REESE VAUGHN AND MAX LEVINE

Iowa Engineering Experiment Station, Iowa State College, Ames, Iowa

Received for publication February 24, 1936

I. HISTORICAL

Gayon (1877) was probably the first to demonstrate the formation of H_2S by bacterial action. He employed lead acetate as an indicator. Since that time the phenomenon of H_2S production by bacteria has received considerable attention.

The production of H_2S in a suitable medium has long been used as a means of differentiation of species in the colon-typhoid group of bacteria. Orlowski (1895) reported the blackening of lead acetate in a peptone medium by *Eberthella typhosa* but not by *Escherichia coli*. Sacquépée and Chevrel (1905) confirmed the work of Orlowski and noted further that paratyphoid B blackened lead acetate, iron potassium tartrate and nickel sulfate whereas paratyphoid A did not.

Burnet and Weissenbach (1915) confirmed the results of Sacquépée and Chevrel and advocated the use of lead acetate agar for differentiating paratyphoid A and B.

Jordan and Victorson (1917), Kligler (1917) and others used and recommended lead acetate agar media for detecting H_2S formation by colon-typhoid bacteria. Wilson (1923) employed a ferric chloride medium to detect sulfides as did Beckwith and Moser (1932).

Levine and co-workers (1932), in an attempt to develop a medium better suited for use with the colon-aerogenes group of bacteria than the lead acetate media previously referred to, employed nickel, iron, lead and manganese salts as indicators of H_2S in peptone media. They suggested an agar medium which contained 2.0 per cent proteose peptone (Difco) with 0.05 per cent ferric citrate as the indicator. This medium was particu-

larly suitable for the detection of H_2S production by members of the colon-aerogenes group. It was pointed out by Levine *et al.* that the phenomenon of H_2S production from proteose peptone might possibly be a means of differentiating the members of the colon-aerogenes "Intermediates" (genus *Citrobacter*) from the genera *Escherichia* and *Aerobacter*.

Pacheco and Mello (1932) reported an agar medium containing 3.0 per cent Witte peptone, with 0.05 per cent bismuth carbonate as an indicator, to be satisfactory for the detection of H_2S produced by *Proteus vulgaris*.

Patrick and Werkman (1933) employed a medium containing 2.0 per cent proteose peptone (Difco) and 0.1 per cent cysteine with ferrous chloride (0.1 per cent) as an indicator of H_2S production. These workers used the medium in connection with a detailed study of bacteria fermenting xylan.

ZoBell and Feltham (1934) compared lead, bismuth and iron as detectors of H_2S produced by bacteria and recommended that either 0.05 per cent ferric ammonium citrate or 0.03 per cent ferrous acetate be employed as an indicator for H_2S in solid media containing at least 3.0 per cent bacto-tryptone (Difco). However, for testing new and unknown pure cultures of bacteria for their H_2S-producing properties these workers advocated the use of lead acetate papers and a suitable liquid peptone medium.

The Digestive Ferments Company Iron Peptone Agar, a modified formula of Levine *et al.* (1932) containing 2.0 per cent bacto-tryptone with 0.05 per cent ferric ammonium citrate as the indicator, has been recommended for detection of H_2S.

The purpose of this investigation was to determine the relative usefulness of the various solid media recently recommended for the detection of H_2S formation by members of the colon-aerogenes group of bacteria and to observe the effect of certain factors on the formation of H_2S from proteose peptone by these organisms.

II. PRODUCTION OF HYDROGEN SULFIDE BY BACTERIA OF THE COLON-AEROGENES GROUP

Preliminary investigation of the medium of Pacheco and Mello indicated that it was not satisfactory for detection of H_2S pro-

duction by the strains of bacteria studied, as test organisms known to be markedly positive in other solid media either failed to give evidence of H_2S production or the reaction was faint.

The media considered below are those of Patrick and Werkman (1933), the dehydrated peptone iron agar of the Digestive Ferments Company, two media containing 3.0 per cent bacto-tryptone (one with 0.05 per cent ferric ammonium citrate and

TABLE 1

Composition of media

CONSTITUENTS	MEDIUM 1*	MEDIUM 2	MEDIUM 3	MEDIUM 4	MEDIUM 5	MEDIUM 6	MEDIUM 7
	grams per liter	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*
Proteose peptone	20.0	20.0	20.0				
Bacto-tryptone					20.0	30.0	30.0
Cysteine		1.0	1.0	1.0			
Agar	15.0	5.0	5.0	5.0	15.0	15.0	15.0
Glucose		1.0					
Ferrous chloride		1.0	1.0	1.0			
Ferric ammonium citrate					0.5	0.5	
Ferric citrate	0.5						
Ferrous acetate							0.3
K_2HPO_4	1.0	0.3	0.3	0.3	1.0	1.0	1.0
$(NH_4)_2SO_4$				0.3			
$(NH_4)_2HPO_4$				0.7			
$CaCl_2$				0.2			

* 1, Levine *et al.* (1932); 2–4, Patrick and Werkman (1933); 5, Digestive Ferments Co.; 6 and 7, ZoBell and Feltham (1934).

the other with 0.03 per cent ferrous acetate) as recommended by ZoBell and Feltham (1934), and the control medium of Levine and co-workers (1932).

The compositions of the test media are shown in table 1.

The constituents for media 1, 5, 6 and 7 were dissolved in boiling water, tubed and sterilized at 15 pounds pressure for fifteen minutes.

For preparation of the media containing cysteine (nos. 2, 3 and 4) it was found necessary to sterilize the indicator (ferrous

chloride) and the other constituents separately. The two sterile solutions were mixed and distributed into sterile tubes under aseptic conditions.

The medium of Patrick and Werkman (medium 2) contains glucose and cysteine in addition to proteose peptone. Seiffert (1909) reported that glucose decreased H_2S production by *Salmonella schottmuelleri*. Myers (1920), Muslow and Paine (1925) and others have shown that organic sulfur compounds of known composition (cystine) can be attacked by the colon group with the formation of H_2S. Tilley (1923) reported that the addition of cystine to lead acetate agar containing peptone resulted in the production of H_2S by *Escherichia coli* and other organisms usually negative.

It was thought that the glucose and cysteine in the medium of Patrick and Werkman would independently influence the production of H_2S. Medium 3 is identical with no. 2 except that glucose was not employed and medium 4 is a synthetic cysteine medium.

The reactions of the finished media were between pH 6.8 and pH 7.0. Preliminary investigation of the effect of reaction on H_2S production in medium 1 using twenty "Intermediate" test strains indicated that H_2S production was distinctly retarded at a reaction of pH 6.0. At reactions of pH 6.5 to pH 8.0 the rate of H_2S production, as indicated by blackening of the medium, was apparently normal.

These seven media were inoculated from twenty-four-hour broth cultures by stabbing into the middle of the solidified medium and also down the side of the medium in contact with the inner surface of the tube. Incubation was at 37°C.

Six hundred and seventy-two colon-aerogenes strains including 229 *Escherichia*, 299 *Aerobacter* and 144 "Intermediate" cultures were observed for H_2S production. The results are summarized in table 2.

Over 95 per cent of the *Escherichia* strains, 100 per cent of the "Intermediate" strains and 77 per cent of the *Aerobacter* cultures produced H_2S from cysteine. The production of H_2S in media containing cysteine is of no differential significance for members of the colon group.

Comparing media 2 and 3, it was noted that in the medium containing glucose the production of H_2S, by the *Aerobacter* strains was definitely retarded. Observations with twenty "Intermediate" strains disclosed that a concentration of 0.01 per cent glucose in medium 1 had no effect whereas concentrations of 0.05 and 0.1 per cent, or more, of glucose, markedly retarded production of H_2S.

TABLE 2

Evidence of H_2S production in various culture media

MEDIUM	AGAR	GENUS							
		Escherichia		"Intermediate"		Aerobacter			
						cloacae		aerogenes	
		229 strains		144 strains		105 strains		194 strains	
		Days at 37°C.							
		2	4	2	4	2	4	2	4
		Per cent producing H_2S							
	per cent								
1	1.5	1.8	2.2	76.4	76.4	0.0	0.0	0.0	0.0
2	0.5	98.7	98.7	99.3	100.0	65.8	73.4	76.3	92.7
3	0.5	98.7	98.7	100.0	100.0	78.1	81.9	97.4	97.4
4	0.5	93.5	95.2	100.0	100.0	71.4	74.3	66.5	74.2
5	1.5	0.9	1.3	60.4	69.5	0.0	0.0	0.0	0.0
6	1.5	0.9	1.3	42.3	49.3	0.0	0.0	0.0	0.0
7	1.5	0.4	1.3	20.8	39.6	0.0	0.0	0.0	0.0

Comparing media 1, 5, 6 and 7, it was noted that more "Intermediate" strains produced H_2S in the proteose peptone medium (no. 1) of Levine *et al.* than in the media containing Bacto-tryptone (nos. 5, 6 and 7) as the source of sulfur.

III. EFFECT OF CONCENTRATION OF PROTEOSE PEPTONE AND BACTO-AGAR ON HYDROGEN SULFIDE PRODUCTION

The proteose peptone, ferric citrate medium (no. 1) seemed most desirable for use. In view of the fact that a concentration of at least 3.0 per cent bacto-tryptone was recommended by

ZoBell and Feltham, and in the cysteine media (nos. 2, 3 and 4) only 0.5 per cent agar was present, the influence of concentration of proteose peptone (Difco) and bacto-agar was investigated.

A. Effect of concentration of proteose peptone

For the purpose of ascertaining the influence of concentration of proteose peptone on the production of H_2S by colon-aerogenes strains, medium 1 with concentrations of 2.0, 3.0, and 5.0 per cent proteose peptone was employed. The three media were tubed, sterilized and inoculated as described above. The results with the six hundred seventy-two coli-aerogenes strains previously referred to are summarized in table 3.

TABLE 3

Effect of concentration of proteose peptone on H_2S production by bacteria of the colon-aerogenes group*

CULTURES	CONCENTRATION OF PEPTONE					
	2.0 per cent		3.0 per cent		5.0 per cent	
	Days incubation at 37°C.					
	2	4	2	4	2	4
	Per cent producing H_2S					
Escherichia 229 strains	1.8	2.2	1.8	2.2	1.8	2.2
Intermediate 144 strains	76.4	76.4	76.4	76.4	76.4	76.4
Aerobacter cloacae 105 strains	0.0	0.0	0.0	0.95	0.95	12.4
Aerobacter aerogenes 194 strains	0.0	0.0	0.0	0.0	0.0	6.7

* Other constituents as in medium 1.

There was no difference observed in the rate of production of detectable amounts of H_2S by the *Escherichia* and "Intermediate" strains in the various concentrations of proteose peptone studied but on long incubation (four days) a few *Aerobacter* strains (12.4 per cent *A. cloacae* and 6.7 per cent *A. aerogenes*) became positive in the higher (5 per cent) peptone concentration.

B. Effect of concentration of bacto-agar

A medium identical with medium 1 but containing various quantities of agar (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 per cent),

was employed to ascertain the influence of concentration of agar on H_2S production. The resulting media were prepared and inoculated as described above. The six hundred and ninety-seven colon-aerogenes strains investigated included 229 *Escherichia*, 169 "Intermediate" and 299 *Aerobacter* strains.

The results are shown in table 4.

TABLE 4

Effect of concentration of agar on H_2S production by bacteria of the colon-aerogenes group*

AGAR	GENUS							
	Escherichia		"Intermediate"		Aerobacter			
					cloacae		aerogenes	
	229 strains		169 strains		105 strains		194 strains	
	Hours at 37°C.							
	24	48	24	48	24	48	24	48
	Per cent producing H_2S							
per cent								
0.0	58.1	93.5	74.6	91.1	1.9	43.8	2.1	74.7
0.5	19.7	37.6	71.0	74.0	0.0	0.95	0.0	0.0
1.0	1.8	16.2	70.4	74.0	0.0	0.0	0.0	0.0
1.5	1.8	1.8	69.8	74.0	0.0	0.0	0.0	0.0
2.0	1.8	1.8	65.1	74.0	0.0	0.0	0.0	0.0
2.5	1.8	1.8	59.2	72.8	0.0	0.0	0.0	0.0
3.0	1.8	1.8	56.8	72.2	0.0	0.0	0.0	0.0

* Other constituents as in medium 1.

In a medium containing 2 per cent proteose peptone, with 0.05 per cent ferric citrate as the indicator, it was observed that the concentration of agar very markedly influenced the rate of detection of hydrogen sulfide production. Thus, with the medium containing 1.5 per cent agar, 74 per cent of the "Intermediate" strains gave positive hydrogen sulfide reactions, whereas less than 2 per cent of the *Escherichia* and none of the *Aerobacter* strains were positive. Reducing the concentration of agar in the

medium resulted in a marked increase in the number of positive reactions, which rose to 37.6 per cent, among the *Escherichia* strains, whereas there was no increase in the number of positives among the "Intermediate" strains and only 1 per cent of the *Aerobacter* strains now reacted positively for hydrogen sulfide.

Eliminating the agar entirely from the medium, that is, employing the liquid, it was found that over 90 per cent of *Escherichia* and "Intermediate" strains showed the black coloration characteristic of hydrogen sulfide production, and what is particularly striking was the increase in positive reactions to 74.7 per cent for the *A. aerogenes* and 43.8 per cent for the *A. cloacae* or 64.0 per cent for all of the *Aerobacter* strains observed.

It is apparent from these results that the concentration of agar is a very vital factor as respects the differential value which the medium originally reported by Levine and his associates may have. The cause of this influence of agar has not been definitely ascertained.

IV. SUMMARY

Practically all *Escherichia* as well as the "Intermediate" strains produced H_2S from cysteine. Over 75 per cent of the *Aerobacter* strains possess this characteristic. The production of H_2S in media containing pure cysteine in addition to peptone is not considered of differential significance in classification of the colon group.

The concentration of proteose peptone was not a significant factor as respects the utilization of H_2S production as a differential test in the colon-aerogenes group of bacteria.

The concentration of agar was a very significant factor as respects utilization of H_2S production for differentiation of colon-aerogenes bacteria. In a medium consisting of 2.0 per cent proteose peptone (Difco), 1.5 per cent agar (Difco), 0.1 per cent K_2HPO_4 and 0.05 per cent ferric citrate, H_2S was produced by 74.0 per cent of the "Intermediate," 1.8 per cent of the *Escherichia* and none of the *Aerobacter* strains. In the absence of the agar the medium lost its differential value as positive reactions were obtained with 91.1 per cent of the "Intermediate," 93.5 per cent of the *Escherichia* and 64 .0 per cent of the *Aerobacter* strains.

When the production of hydrogen sulfide is employed as a differential test in the colon group, it is not sufficient to record merely production or failure to produce this end-product, but it is imperative that the constituents of the medium and the period and temperature of incubation be carefully designated.

REFERENCES

BECKWITH, T. D., AND MOSER, J. R. 1932 Jour. Bact., **24**, 43–52.

BURNET, ÉT., AND WEISSENBACH, R. J. 1915. Compt. rend. de Soc. Biol., **78**, 565–568.

GAYON, U. 1877 Note de M. U. Gayon présentée par M. Pasteur. Comp. rend. Acad. Sci., **85**, 1074–1076.

JORDAN, E. O., AND VICTORSON, RUTH. 1917 Jour. Infect. Dis., **21**, 554–555.

KLIGLER, I. J. 1917 Amer. Jour. Pub. Health, **7**, 1042–1044.

LEVINE, MAX, VAUGHN, REESE, EPSTEIN, S. S., AND ANDERSON, D. Q. 1932 Proc. Soc. Exp. Biol. Med., **29**, 1022–1024.

MYERS, JOHN T. 1920 Jour. Bact., **5**, 231–252.

MUSLOW, FREDERICK W., AND PAINE, FREDERICK S. 1925 Proc. Iowa Acad. Sci., **32**, 64–70.

ORLOWSKI, M. A. 1895 Jour. Med. Milit. Russe. After Jahresber. Path. Mikroörgan., **11**, 528–529.

PACHECO, G., AND MELLO, T. 1932 Annaes da Faculdade de Med. de Sao Paulo, **8**, 3–10.

PATRICK, ROGER, AND WERKMAN, C. H. 1933 Iowa State College Jour. Sci., **7**, 404–418.

SACQUÉPÉE, E., AND CHEVREL, F. 1905 Compt. rend. Soc. Biol. **59**, 535–536.

SEIFFERT, G. 1909 Zeit. f. Hyg. **63**, 273–290.

TILLEY, F. W. 1923 Jour. Bact., **8**, 115–120.

WILSON, W. J. 1923 Jour. Hyg., **21**, 392–398.

ZOBELL, C. E. AND FELTHAM, C. B. 1934 Jour. Bact., **28**, 169–176.

ULTRA-VIOLET IRRADIATED CARBOHYDRATES AND BACTERIAL GROWTH

JOHN GEORGE BAUMGARTNER

Crosse and Blackwell, Limited, Crimscott Street, Bermondsey, London, England

Received for publication February 29, 1936

The following experiments were undertaken in an endeavour to confirm the work of Blank and Arnold.[1] These authors make the following assertions:

1. Exposure of any one of 20 different carbohydrates and 3 carbohydrate derivatives to U.V. light, (2537 Å) for periods varying from 1.0 to 5.0 hours, results in the growth inhibition of *Bacillus subtilis* spores, when such irradiated carbohydrate solutions are incorporated in the medium.
2. Similar irradiation of agar and agar-water gels so alters them that when used as a base for culture media, such media do not support the growth of *Bacillus subtilis* spores.
3. This results from the formation of a non-volatile, thermostable material, capable of diffusing through and from an agar gel, which prevents the growth of the organism.

Using as far as possible, the same technique as these authors and a mercury vapour lamp[2] giving 99 per cent of the emission in the line 2536 Å the results of our experiments were as follows:

Experiment 1. Irradiation of entire agar culture medium

An agar plate containing 10 ml. of sterile nutrient agar was irradiated for four hours at a distance of 5 cm. from the source of light. The pH of this medium was 7.2 before exposure. After irradiation the pH of the medium was found to be 4.6. Inoculation of this plate with spores of *Bacillus subtilis* and *Escherichia coli* was not followed by colony development within 72 hours. If, however, a plate is irradiated and

[1] Blank, I. H. and Arnold, W.: Jour. Bact., 30, 507, 1935.
[2] Vi-Tan Ultra Violet Lamp, Manufactured by The Thermal Syndicate, Ltd.

before inoculation the pH is adjusted to 7.0, growth occurs with both types in 24 hours.

Experiment 2. Irradiation of agar gel

When 20 ml. of 2 per cent agar-water gel was irradiated for 3 hours, it was found that the pH of the gel dropped from 7.0 to about 4.0. This gel was used to prepare 20 ml. of nutrient agar, and the pH after sterilisation was 4.5 to 4.6. This medium did not permit growth of *Bacillus subtilis* spores or *Escherichia coli*. Adjustment of the pH of such medium to 7.0 resulted in colony formation by both types within 24 hours.

Experiment 3

Blank and Arnold state that if an irradiated agar gel is soaked in water, the gel removed and the soak water evaporated to dryness on a steam bath and the resulting solids incorporated in a culture medium, such medium does not support the growth of *Bacillus subtilis*.

We have found that a medium prepared in this fashion will have a final pH of about 4.6 to 4.8, and does not support the growth of *Bacillus subtilis*. If the medium is neutralised, however, growth occurs readily.

Experiments in which sucrose was irradiated have given similar results.

Experiment 4. Irradiation of 1 per cent sucrose

We have observed that irradiation of 1 per cent sucrose solution for 2 hours resulted in a drop in pH from 7.0 to 3.5–4.0. The use of such solutions as a base for nutrient media without subsequent pH adjustment was naturally followed by inhibition of the growth of bacteria.

If, however, the medium is neutralised before use, growth occurs readily.

Experiment 5. Irradiation of 1 per cent sucrose in the presence of excess of $CaCO_3$

If 1 per cent sucrose solution is irradiated in the presence of an excess of $CaCO_3$, there is little or no change in the pH of the solution and media prepared from it will readily support bacterial growth without any pH adjustment. This would seem to indicate that the sole inhibitive agent formed by the irradiation of carbohydrates is the acidity.

Our investigation has shown that approximately half the acidity formed by irradiation of a 1 per cent sucrose solution for 2 hours is due to formic acid. The remainder consists of non-volatile organic acids. Traces of formaldehyde were also present.

It should be mentioned that Blank and Arnold make no reference to the pH of their media before or after irradiation. They state that the question of the influence of H-ion concentration of the carbohydrate solution during irradiation still requires investigation (presumably in relation to the quantitative course of the reaction producing the inhibitory substance).

CONCLUSIONS

1. Confirmation has been obtained of the claim by Blank and Arnold that ultra-violet radiation (2357 Å) causes agar gels or sucrose solutions when subsequently included in culture media to inhibit the growth of *Bacillus subtilis* and *Escherichia coli*.
2. Such radiation of carbohydrates is accompanied by marked production of acid.
3. Approximately half this acid is formic acid.
4. Neutralisation of this acidity restores the ability of the culture media to support the growth of the bacteria.

The author gratefully acknowledges the interest shown by Dr. William Clayton in this investigation.

CLOSTRIDIUM BOTULINUM TYPE C. IN RELATION TO DUCK SICKNESS IN THE PROVINCE OF ALBERTA

R. M. SHAW AND GRETTA S. SIMPSON

Provincial Laboratory, University of Alberta, Edmonton, Alberta

Received for publication March 13, 1936

During the summer of 1933 a severe epidemic occurred among wild ducks feeding about Stobart and Namaka lakes, situated in the southern part of the Province of Alberta, Canada, about 45 miles east of the city of Calgary and 130 miles north of the Montana border. Thousands of birds died during the outbreak. Sportsmen became greatly concerned and wide publicity was given to the epidemic through the newspapers. Many theories were advanced in explanation. The observed symptoms in the affected birds were those of what has come to be known as western duck sickness.

Kalmbach, in 1930, while making field studies in duck sickness in the United States, directed attention to the probability of bacterial toxins playing an etiological rôle. His findings together with a review of the history of the disease were published by him in collaboration with Gunderson in 1934.

In 1930 Giltner and Couch isolated *Clostridium botulinum*, type C. from the organs of diseased wild fowl. The same organism was later isolated by Gunderson and by Hobmaier (1930) under similar circumstances. The presence of toxin has been demonstrated in the feeding grounds in mud and water and in the bodies of larvae of beetles. It appears to be generally rather widely distributed in localities where duck sickness is prevalent (Kalmbach and Gunderson, 1934).

We became interested in the 1933 Alberta outbreak when on September eighteenth we received, through the courtesy of Dr. William Rowan, Professor of Zoölogy, University of Alberta, eleven dead waterfowl and samples of mud, weeds and water collected in the epidemic area.

In our study of dead birds and other material submitted, our efforts were directed towards (1) the isolation and study of pure cultures, (2) the demonstration of toxins in the organs of dead birds, mud, water or cultures of these and (3) the comparison of our organism with a known culture of *Clostridium botulinum* type C. by toxin-antitoxin experiments.

TECHNIQUE

The bodies of ducks were placed in the incubator at 30° to 35°C. for three to six days. Cultures were made from liver and breast on cooked meat media. A portion of the liver, and in some cases a portion of the breast, was set aside for determination of toxicity.

For the estimation of toxicity of cultures and organ extracts pigeons were mainly used as experimental animals. Some experiments were also conducted on a few wild ducks and guinea pigs. Pigeons were found to be highly susceptible to intramuscular injection though quite resistant and irregular in response to identical toxins given by mouth. In consequence of this, most of our experimental work on toxicity and antitoxin protection was carried out by parenteral inoculation of pigeons.

PURE CULTURES

Stroke plates were made from original cultures and incubated in an atmosphere of hydrogen. Colonies were picked into cooked meat media. After incubation, each was tested for toxicity on pigeons.

By repeated plating and testing finally a culture was obtained from which all picked colonies gave cultures which were uniformly toxic and in which the organisms agreed in morphology and cultural reactions. The symptoms were those of western duck sickness and are reported in detail later.

MORPHOLOGY OF OUR STRAIN NO. 143

When grown on cooked meat medium, at 37.5°C. the bacterium is a large bacillus measuring 4 to 8 microns in length by 0.8 micron in width, with rounded ends. It occurs mainly in singles and

pairs, and occasionally in short chains. It is non-capsulated. Spores are formed readily in cooked meat. They are oval, subterminal and produce moderate bulging of the sides.

TABLE 1

Showing neutralizing effect of antitoxin no. 143 on homologous pure toxin

NUMBER OF PIGEONS USED	TOXIN NO. 143	AMOUNT OF ANTITOXIN NO. 143	REMARKS
		Pigeons inoculated on June 11, 1934, at 5 p.m.	
	cc.	*cc.*	
1	0.001	0.125	No ill effects
1	0.001	0.25	No ill effects
1	0.001	0.50	No ill effects
1	0.001	0.75	No ill effects
1	0.001	1.0	No ill effects
1 control	0.001	None	June 13—weakness of wings June 14—comotose June 15—moribund, chloroformed
		Pigeons inoculated on June 16, 1934	
1	0.02	0.125	No ill effects
1	0.01	0.125	No ill effects
1	0.004	0.125	No ill effects
1	0.002	0.125	No ill effects
1	0.001	0.125	No ill effects
1 control	0.0001	None	June 18—weakness of wings; unable to fly; diarrhea June 19—same June 22—moribund, chloroformed

Antitoxin no. 143 refers to the antitoxin made by inoculating rabbits with toxin from our own pure strain, no. 143, isolated from a mallard duck.

The bacteria stain readily by ordinary dyes and are gram-positive in young cultures. In older cultures, many gram-negative forms are seen.

The organism is a strict anaerobe and does not grow profusely.

FERMENTATION REACTIONS

Incubation of fermentation tubes was under an atmosphere of hydrogen at 37.5°C. Media were enriched by the addition

of 10 per cent human serum. Observations were made after twelve and twenty-eight days incubation. Gas was formed in glucose, galactose, levulose, dextrin and maltose. There was no fermentation of sucrose, lactose, mannitol or salicin. Litmus milk was peptonized and gelatin liquefied. In cooked meat there was very slight production of gas, a rancid odour and very little clouding of the supernatant broth.

TOXICITY EXPERIMENTS

Sterile filtrates from the mud, weeds, and water, in the uncultivated state, when injected in 10 cc. quantities intramuscularly into pigeons failed to produce any toxic symptoms. As will appear later, cultures from the same mud showed *Clostridium botulinum* toxin, indicating that the organisms were nevertheless present. Special technique had not been employed in the collection of samples in the field to obtain concentrated toxin.

Cooked meat media were used for the production of toxin in cultures. Incubation was at 37.5°C. for eight to fifteen days.

Extracts of organs were prepared by grinding the weighed tissue in a mortar with physiological saline, 5.0 cc. per gram of tissue.

Unfiltered material was used primarily in determining the toxicity of cultures but for all other experimental work cultures and organ extracts were sterilized by filtration through a Mandler filter.

The strain of *Clostridium botulinum* encountered in this investigation produces a potent toxin. Many of our culture filtrates and some of the organ extracts were found to produce fatal results in pigeons in 0.0001 cc. quantities.

The following outline indicates the usual trend of events as observed in inoculated pigeons. The incubation period varies with the dosage: those receiving large doses begin to show symptoms in a few hours, some as early as three and one-half hours; while those receiving minute amounts only develop symptoms after days, some as late as six and one-half days.

The first symptoms observed are those of inactivity, the birds having a tendency to sit down. This is followed by weakness of

TABLE 2

Showing neutralizing effect of antitoxin no. 143 on toxic filtrates from impure cultures and liver extracts from our various water fowl

NUMBER AND KIND OF EXPERIMENTAL BIRDS INOCULATED	TOXIN FILTRATES, AMOUNT	ANTITOXIN NO. 143, AMOUNT	REMARKS
	cc.	cc.	
Culture filtrate, mallard duck no. 1 (pure culture filtrate no. 143):			
6 pigeons	0.01	0.25	Five pigeons showed no ill effects. One found dead on sixth day
6 pigeons—controls	0.002	None	Five died in five days. One became ill but recovered
1 wild duck	0.10	1.75	No ill effects
1 wild duck—control	0.10	None	Developed weakness and drooping wings, recovered
Culture filtrate, ruddy duck no. 3:			
3 pigeons	0.01	0.25	No ill effects
3 pigeons—controls	0.002	None	All dead within seven days
Culture filtrate from duck no. 4:			
3 pigeons	0.10	0.33	No ill effects
3 pigeons—controls	0.01	None	All dead within four days
Culture filtrate from duck no. 8:			
3 pigeons	0.10	0.33	No ill effects
3 pigeons—controls	0.01	None	All dead within four days
Liver extract filtrate, green winged teal no. 9:			
2 pigeons	0.01	0.25	No ill effects
2 pigeons—controls	0.002	None	Developed weakness, unable to fly, recovered
Culture filtrate, pintail duck no. 10:			
3 pigeons	0.01	0.25	No ill effects
3 pigeons—controls	0.002	None	All dead within six days
1 wild duck	0.001	0.25	No ill effects
1 wild duck—control	0.001	None	Developed weakness and drooping of wings, recovered

TABLE 2—*Concluded*

NUMBER AND KIND OF EXPERIMENTAL BIRDS INOCULATED	TOXIN FILTRATES, AMOUNT	ANTITOXIN NO. 143, AMOUNT	REMARKS
Culture filtrate, plover no. 11:			
3 pigeons	0.01	0.25	No ill effects
3 pigeons—controls	0.002	None	All dead within six days
1 wild duck	0.01	0.25	No ill effects
1 wild duck—control	0.01	None	Developed drooping of wings. Recovered
1 wild duck	0.10	1.75	No ill effects
1 wild duck—control	0.10	None	Died within six days
Culture filtrate from mud:			
2 pigeons	0.10	0.30	No ill effects
2 pigeons—controls	0.10	None	Both died in two days

Antitoxin no. 143 refers to the antitoxin made by inoculating rabbits with toxin from our own pure strain, no. 143, isolated from a mallard duck.

the wings, which develops rapidly in fatal cases with large doses but more slowly in those receiving smaller amounts. At first the bird is able to lift himself off the ground a few feet but is unable to maintain flight. As the disease progresses lower and lower elevations are attained in attempts to fly until later the wings are used weakly, only as an aid in running. When this stage is reached the bird has also developed great weakness of the legs and frequently tumbles when attempting to run. Early in the disease the nictitating membrane becomes paralysed, the pupils dilate and the lids are not used and often droop, though vision still remains.

Diarrhea, too, is an early symptom, the dejecta having usually a greenish color. With some birds a thick viscid mucous drips from the mouth. After they have lost power in the legs and wings the birds frequently attempt to propel themselves by means of their beaks pressed to the floor. At this time the birds are often alert but as the disease progresses their heads drop forward and coma develops. Breathing becomes slow, shallow and almost imperceptible. A number of birds, with small though fatal doses of toxin, lay for several days in this comatose state. With large doses of a strong toxin, death resulted in some cases within

five hours after inoculation and within two hours after the first observed symptom.

Many inoculation experiments with toxins from liver extracts and cultures were conducted on pigeons and some on wild ducks. It does not appear that any advantage would be gained by relating these in detail. It seems to us sufficient to state that in all those cases where toxicity was observed, the symptoms produced were those of duck sickness (see table 2).

FEEDING EXPERIMENTS

Six pigeons were given a sterile toxic liver extract by means of a tube into the crop as follows: two birds received 2.0 cc. each; two, 5.0 cc. each and two, 10.0 cc. each. One of those that received 2.0 cc. and one that received 10.0 cc. died within eight and two days, respectively. The other pigeons became ill, developed paralysis of the nictitating membrane, weakness of wings and legs and diarrhea but recovered and were able to fly short distances within fourteen days.

Two mallard ducks in excellent condition, obtained from a sanctuary almost two hundred miles from the region of the epidemic, and some months after the outbreak had subsided, were fed 1.0 cc. and 2.0 cc. respectively of a toxic culture filtrate (0.0001 cc. fatal for pigeons intramuscularly) in rolled oats. Both birds developed typical symptoms and died within forty-eight hours.

Guinea pigs fed 2.0 cc. and 5.0 cc. quantities of toxic culture filtrate in rolled oats became very ill within twenty-four hours and died within forty-eight hours, while guinea pigs given similar amounts of the same toxin heated in boiling water for ten minutes suffered no ill effects.

TOXIN-ANTITOXIN EXPERIMENTS

Liver extracts and cultures from seven of the eleven water-fowl, when inoculated into pigeons, gave symptoms typical of those seen in duck sickness. Pure cultures were obtained from one duck only. In order to prove the presence of the same type of organism in the mud and in the remaining six water-fowl, from

which toxic liver extracts and cultures were obtained, without employing the more difficult technique of isolating pure cultures, we proposed to demonstrate its presence by the use of toxin-antitoxin experiments.

Antitoxin was prepared by repeated hypodermic injections into a rabbit, first of toxoid, and later of toxin, both obtained from our own pure culture, no. 143. This serum in 0.125 cc. quantities protected pigeons against 0.02 cc. of toxin, the fatal dose of which, to like birds, was 0.0001 cc.

In these tests for determining the titre of the serum, as well as in all other toxin-antitoxin experiments, the following technique was used. Measured volumes of serum and toxin were mixed and these mixtures, together with suitable dilutions of toxin for control pigeons, were heated in a water bath at 37.5°C. for one-half hour. Immediately following this incubation, pigeons were inoculated intramuscularly. The amount of toxin administered to controls never exceeded the smallest amount used in the accompanying toxin-antitoxin mixture and usually was one-fifth or one-tenth of this amount.

Similar experiments were conducted with cultures made from mud. In all these, control pigeons developed symptoms characteristic of duck sickness and died or were killed when moribund, while those receiving toxin-antitoxin mixtures remained unaffected except in the case of one bird.

COMPARATIVE EXPERIMENTS WITH A KNOWN CULTURE OF CLOSTRIDIUM BOTULINUM TYPE C. NO. 526

Through the kindness of Miss Janet B. Gunnison of the University of California, we obtained a known culture of *Clostridium botulinum* type C. This organism, no 526, was isolated by Graham in 1923 from the stomach of a horse, dead from botulism. In toxin-antitoxin experiments, antitoxin made against our own culture no. 143 protected pigeons against ten times the amount of toxin no. 526 used with fatal results in controls. For results see table 3.

PARASITES

Since leeches occupied a prominent place in the newspapers, in relation to the epidemic, a careful search for these organisms was made. However, only four were found. One mallard had two leeches attached, one in the posterior nares and one just within the nostril. Two other birds had one each in the posterior nares.

Kalmbach in a few paragraphs devoted to a consideration of parasitism in relation to duck sickness makes no reference to filarial worms. An examination of the heart's blood of two ducks killed by us, and, that of one received a few minutes after death,

TABLE 3

Showing neutralizing effects of antitoxin no. 143 on toxin of known organism Clostridium botulinum type C. no. 526

Pigeons inoculated on August 3, 1934

NUMBER OF PIGEONS USED	TOXIN OF CLOSTRIDIUM BOTULINUM TYPE C. NO. 526	ANTITOXIN	REMARKS
	cc.	cc.	
2	0.01	0.125	No ill effects
2	0.01	0.25	No ill effects
3 controls	0.01	None	Two died in four days, one in five days
6	0.1	0.125	No ill effects
1 control	0.01	None	Coma in twenty-four hours. Moribund in two days. Chloroformed

Antitoxin no. 143 refers to the antitoxin made by inoculating rabbits with toxin from our own pure strain, no. 143, isolated from a mallard duck.

revealed the presence in all three of numerous filarial worms, each about 100 microns in length and very active. Four other ducks from quite a different area 200 miles distant and killed many months later showed none of these parasites.

No search was made for parasites in the respiratory or digestive tracts.

SUMMARY

Pure cultures of *Clostridium botulinum* type C. were isolated from the organs of a duck dead of duck sickness.

Antitoxin produced against the toxin of this freshly isolated strain protected experimental birds against homologous toxin and against that of a known culture of *Clostridium botulinum* type C.

The use of this antitoxin in toxin-antitoxin protection experiments enabled us to demonstrate the presence of *Clostridium botulinum* type C. in the bodies of six other water-fowl, and in cultures of mud from the epidemic area.

Tables were prepared showing the neutralizing effect of the antitoxin on toxin from our pure culture strain and from a known strain of *Clostridium botulinum* type C.

Where it was possible to obtain heart's blood from the ducks at or shortly after death, an examination was made and large numbers of filarial worms were found. These were not regarded as having any significance in the epidemic.

REFERENCES

GILTNER, L. T., AND COUCH, J. F. 1930 Science (n. s.) **72**, 660.

HOBMAIER, M. 1930 California Fish and Game, **16**, 285–286.

KALMBACH, E. R., AND GUNDERSON, M. F. 1934 Technical Bulletin, No. 411, May 1934, United States Department of Agriculture, Washington, D. C.

THE LIFE CYCLE OF THE ORGANISM CAUSING YEAST MENINGITIS

RAMONA L. TODD AND WALTER W. HERRMANN

Department of Hygiene, Preventive Medicine and Bacteriology, State University of Iowa, Iowa City

Received for publication March 19, 1936

In studying the characteristics of several strains of the organism commonly called *Torula histolytica*, some rather prominent structures were noted, the significance of which seems to have been overlooked in previous descriptions. The study of the nature of these bodies disclosed a sequence of changes in morphology which necessitates a revision of the classification of at least some of the members of this group.

One of the strains studied was isolated on four occasions from the spinal fluid of a patient in the University of Iowa Hospitals. This man had symptoms indicative of lesions in the central nervous system for two years prior to admission in 1934. In January, 1936, his disturbance had neither progressed nor subsided.

Another strain was recovered from the spinal fluid of a patient in the Minneapolis General Hospital in 1935. His illness, from onset to death in three months, was characterized by symptoms referable to the central nervous system and at necropsy these tissues exhibited typical lytic lesions. We are indebted to Dr. A. T. Henrici and Dr. N. H. Lufkin for the culture and history of this latter case.

OBSERVATIONS

Cultures of these two strains of organisms, when studied in the ordinary fashion, exhibited all of the described characteristics of *Torula*: reproduction by budding, no mycelium formed and glucose alone fermented, and without gas formation. However,

in old cultures of both strains structures were found different from the cells seen in young cultures. Outstanding among these were thick-walled cells with a prominent outer shell in various stages of being shed. There was also extreme variation in the size, shape and internal structure of other cells. These variations were studied in van Tieghm cell cultures, and by daily hanging drop preparations of the same cultures over a period of seven weeks. Attempts to follow all of the changes in a single cell have been unsuccessful thus far, but from the various preparations we feel that we have sufficient evidence to describe a life cycle for these organisms. Thus, starting with the organism as it is usually seen in culture from patients: young cells appeared globose to ovoid, and ranged up to 10 μ in diameter, with an average of 6 to 8 μ. The contents were granular, the cell walls distinct and rigid. No ameboid cells were found. Mycelium was not observed in any of the cultures. A capsule surrounded the cell, occasionally appearing as a matrix around several cells. The buds were nearly always single, but now and then a cell with two or three buds was seen.

On Sabouraud's maltose or glucose medium, pH 7.0, incubated at from 20° to 37°C., multiplication by budding proceeded until the growth was about three weeks old. If a moist condition was maintained, the budding continued uninterruptedly for about six weeks or longer. When the medium began to dry out in a relatively old culture, two types of cells, differing slightly, morphologically, appeared. One type was fairly thick-walled (Cell 1), the other thinner-walled (Cell 2), more nearly as in the young budding cells. The former were spherical, had distinct, medium-thick walls, and each cell contained a large, single globule. This globule stained with Sudan III and with osmic acid. These cells were 5 to 8 μ in diameter; rarely they were a little larger. Some cells were seen in these old cultures with short protuberances, varying from a slight bulge of the wall to a definite tube up to 3 or 4 μ long. The thinner-walled cells were spherical to ovoid, the ovoid forms being usually more numerous. The cytoplasm was granular, but with several small, spherical bodies.

Many of these thinner-walled cells also had tubes of varying lengths. Both types of cells were seen with buds (Cells 3 and 4).

On two occasions, in broth cultures observed continuously in van Tieghm cells, set up from a seven weeks old culture on Sabouraud's maltose agar, a process which may be interpreted as fusion was watched. In each instance a cell of the thin-walled type and one of the thick-walled type was involved. The two cells each had short tube-like projections by which they became united. In one such instance, the tube of the thinner-walled cell was seen to meet the thicker-walled cell at a point where only a slight protrusion was observable.

Temporary mounts showed preparations which indicated that the entire contents of the thinner-walled cell had passed through the narrow germ tube into the thicker-walled cell. Various stages of the process were noted in temporary mounts from old cultures on glycerin agar, including many cells connected by means of tubes. In many of these pairs the thin-walled cells were seen in various stages of collapse, the large globules in the opposite cells having disappeared. In some instances, a tube of a collapsed cell was seen still attached to a thick-walled cell, entirely different from the one described above. This cell was composed of a central oval or spherical area filled with fairly small granules, almost completely surrounded by one, and sometimes two, outer layers. The first of these layers was usually in contact with the inner mass except for a small area near one pole. This opening ranged from a minute aperture to complete separation of outer husks and inner oval or spheroid (Cells 17 to 29). The outermost covering was much less extensive and lay as a cap over the end opposite the aperture. Not more than one of the inner spherical or ovoid structures was seen inside the husks or shells. The central area of one of these double-coated forms was observed in a van Tieghm cell: the granules were seen to coalesce and form a homogeneous mass. This mass by a series of divisions was seen to appear first as two, then as four, small, spherical bodies. In temporary mounts, similar cells with more than four such bodies were observed (Cell 17). These

bodies took fat stains. Many of these spheroids, freed from the outer husks, as well as many empty shells, were seen in mounts of very old cultures of the organisms. The freed spheroids were fairly thick-walled structures filled with varying numbers of globules (Cell 30), and ranged from 7 to 11 μ in diameter, with an average of about 9 μ. As many as 22 globules were counted in a single spheroid, while 14 to 18 seemed to be the most common range, but many bodies had fewer than 14 globules. In a single instance, the wall of the cell was seen to rupture and allow the several small globules to scatter into the medium. It is not know whether this was due to the environmental conditions existing in the medium, or to a force such as jarring, or if it was the result of natural development.

These freed cells germinated by budding; usually, a single bud, but sometimes more than one, formed at a time; or the buds formed in succession, and remained attached to the parent cell. The bodies were even seen to bud while the husks were still attached. In general, the freed cells seemed to act much as young vegetative cells.

DISCUSSION

In light of the foregoing observations, it seems logical to interpret the findings as representing the perfect stage of this organism. The young budding cells are similar morphologically, but in old cultures two types of cells are produced (Cells 1 and 2). Both types may bud (Cells 3 and 4). The two kinds of cells are attracted to each other at the proper stage in their development; they fuse, the intervening wall breaks down, and the contents of the thin-walled cell are transferred to the thick-walled cell (Cells 5 to 8). It is logical to assume that the globule which had developed in the recipient cell, and which takes fat stains, is stored food, subsequently used to support the growth and development of the zygote. The fertilization process leaves the wall of the donor cell as a collapsed shell (Cells 9 to 11).

The contents of the zygote coalesce to form a homogeneous mass, more or less elongated, and lying eccentrically in the cell (Cell 13). This cytoplasmic mass is the first phase in the de-

velopment of the single spore. It breaks up into many fat or fat-containing globules which almost fill the developing ovoid spore (Cells 16 to 25). The outermost of the two layers (Cells 17 to 22), seen at this stage, is probably the old wall of the recipient cell. The inner husk is the spore sac. The formed spore may bud even before it has escaped from the spore sac, the bud forming at the aperture (Cells 18 and 24). The spore becomes rotund, and the sac begins to break away, finally being completely shed (Cells 30 and 31). The freed spores germinate by budding, giving rise to the young globose cells (Cells 32 to 34).

In addition to the strains isolated from the Iowa City and the Minneapolis cases, we have observed the same phenomena in subcultures of the Rappaport and Kaplan (1926) organism, the strain isolated by Freeman and Weidman (1923), the one reported by Fitchett and Weidman (1934), that reported by Shapiro and Neal (1925), the strain from Firor's (1926) case, an organism from an unpublished case of Jacobson,[1] a strain of unknown origin received from Dr. W. B. Stovall, and one from the University of Iowa, the source of which could no longer be determined.

No such phenomena have been reported in the literature concerning this species, although structures have been described which would indicate that at least phases of the cycle have been seen. Stoddard and Cutler (1916) mention finding large resting cells. They also describe two different types of cells, a large thick-walled form, and a smaller thin-walled form, the more numerous of the two. They mention the "particles" inside the cells. They found empty capsules, which they say, suggest sporulation, but no other evidence of such a process could be found. Freeman and Weidman (1923) state that their organism in young cultures contained budding cells, the contents of which were coarsely granular; a few days later large cells appeared with a double contoured-wall and a spheroid body which almost filled the interior. They state: "In cultures several weeks old the central part of such a cell contained from two to eight or more

[1] Personal communication from H. P. Jacobson. Organism isolated in Los Angeles, Cal.

larger or smaller hyaloid spheroids. These were variable both in number and size. We, therefore, finally concluded that they were not asci."

Sheppe (1924) describes a peculiar budding process. From micro-cultures, the cells, according to him, acted as follows: "a slight protuberance appeared on the side of the parent cell; this protuberance enlarges and both layers of the cell membrane stretch over the bud, nuclear material passes into the bud, the pedicle of the bud is often considerably elongated, the bud begins to increase in size, the whole representing a curious dumb-bell appearance when it reaches the size of the parent. After about 20 hours at room temperature, the process is completed by the constriction and final severance of the pedicle. This severance leaves both ogranisms pyriform in shape. The rounded contour is gradually reassumed." Benham (1934), in describing *Cryptococcus hominis*, says "in old cultures of a month or more which have begun to dry, large, thick-walled nonbudding cells filled with granules may be seen." Many other writers describe what they call thick-walled resting cells in old cultures.

Sporogenous cells in which meiosis occurs, which form their spores internally through free cell formation, are asci, and the spores formed in such a manner are ascospores. This is the criterion upon which a fungus, possessing such cells, is placed in the class Ascomycetes. The activities of the nuclei of many fungi which bud, lack a true mycelium, and produce a single endogenous spore, either parthenogenetically or as a result of fusion, have not been determined. Such forms are placed in the class Ascomycetes and grouped with the true yeasts, the family Saccharomycetaceae. The cytological activity of these forms, when determined, will prove whether the body interpreted as an ascospore fulfills the requirements or whether meiosis is delayed and it is really an oospore. The strains of organisms which follow the cycle herewith presented must be classified with the single-spored yeasts regardless of the solution of this problem.

The question of the nomenclature and correct terminology for the imperfect yeast-like organisms has long been disputed, and the use of various names for the designation of these organisms

has led to great confusion. Benham (1935 a and b) has investigated these groups of fungi, and concludes that *Cryptococcus* Kützing, 1833, should be adopted as the genus name of "those unicellular fungi consisting of round or ovoid cells occasionally in chains but never forming a well-defined mycelium. Reproduction by one or more buds; no ascospores. Growth on artificial media in pasty or dry colonies, white or colored." She places the genus in the family Pseudosaccharomycetaceae in the order Moniliales of the class Fungi Imperfecti. She bases her selection of *Cryptococcus* as the genus for these organisms on priority of the name and usage among medical mycologists.

Lodder (1934) decides that *Torulopsis* Berlese, 1894, should be the accepted genus name for these fungi. Castellani (1935) also adopts it. The genus *Torula* was created by Persoon (1801) for a fungus with short dark hyphae and catenulate conidia; consequently, *Torula* Turpin, 1838, referring to the yeast-like fungi, is a misnomer and should be discarded on a priority basis.

The question, then, seems to be whether *Cryptococcus* or *Torulopsis* is the valid genus name for this group. Dodge (1935) accepts both names as representing separate, distinct genera. This does not seem to be a justified decision. Benham (1935 b) includes the generic names *Torulopsis*, *Torula*, and *Eutorula* as synonyms of *Cryptococcus;* and suggests that the species *Cryptococcus hominis* Vuillemin, 1901, be adopted as a representative organism of the genus.

It seems advisable to accept on the basis of priority, and not without a consideration of usage, the genus name *Cryptococcus* Kützing, 1833, for these yeast-like organisms, and *Cryptococcus hominis* as the correct species for the non-spore forming organisms of yeast meningitis. *Torula histolytica* Stoddard and Cutler, 1916, *Torulopsis hominis*, and *Saccharomyces tumefaciens* Castellani and Chalmers, 1913, become synonyms of this species.

CLASSIFICATION OF THE SPORULATING "CRYPTOCOCCUS HOMINIS"

While it no doubt is advisable to retain the imperfect genus *Cryptococcus* and the species *hominis* for the strains which either do not show this type of activity or which have not been studied

from the standpoint of spore formation, it is necessary to revise the classification of the strains which do form spores. Since the spore sac contains but a single spore and Kloecker's (1909) description of *Debaryomyces*, 1909, is applicable, this genus is selected. A number of species of pathogenic yeasts have been referred to the genus *Debaryomyces* by Ota (1923, 1924 a and b). Several of these were isolated from skin lesions and were proved to be pathogenic for laboratory animals. The yeasts from American cases of meningitis are apparently identical with the organism from cases of European blastomycosis (Lodder, 1934). The latter, described fully by Busse (1894) and Buschke (1898), may fall in this same genus when its complete life cycle is determined.

Debaryomyces is described by Dodge (1935) as follows: "Cells predominantly spherical but also ovoid, mostly small; vegetative reproduction by sprouting. Asci following isogamous or heterogamous copulation occasionally parthenogenetic; spores spherical mostly with a large central oil globule, wall verrucose (warts often difficult to see), usually only one spore per ascus.... The ascus develops directly in the zygote." The ten strains of organisms of yeast meningitis listed above fit this description. The species name *hominis* should be carried over for the spore-bearing forms, according to the rules of the International Code of Botanical Nomenclature. Therefore, it is suggested that the spore-forming strains of "*Cryptococcus hominis*" (*Torula histolytica*) be called *Debaryomyces hominis* (Vuillemin) Todd and Herrmann *n. comb.* This species is briefly described as follows: vegetative cells globose to ovoid up to 10 μ in diameter, surrounded by a capsule, buds usually single. Copulation heterogamous, ascus developing directly in the zygote, single spored. Spore globose or slightly oval, from 7 to 11 μ in diameter, wall definite and of medium thickness, spore filled with varying numbers of fat or fat-containing globules, spore germinates by budding. Organism pathogenic for humans.

REFERENCES

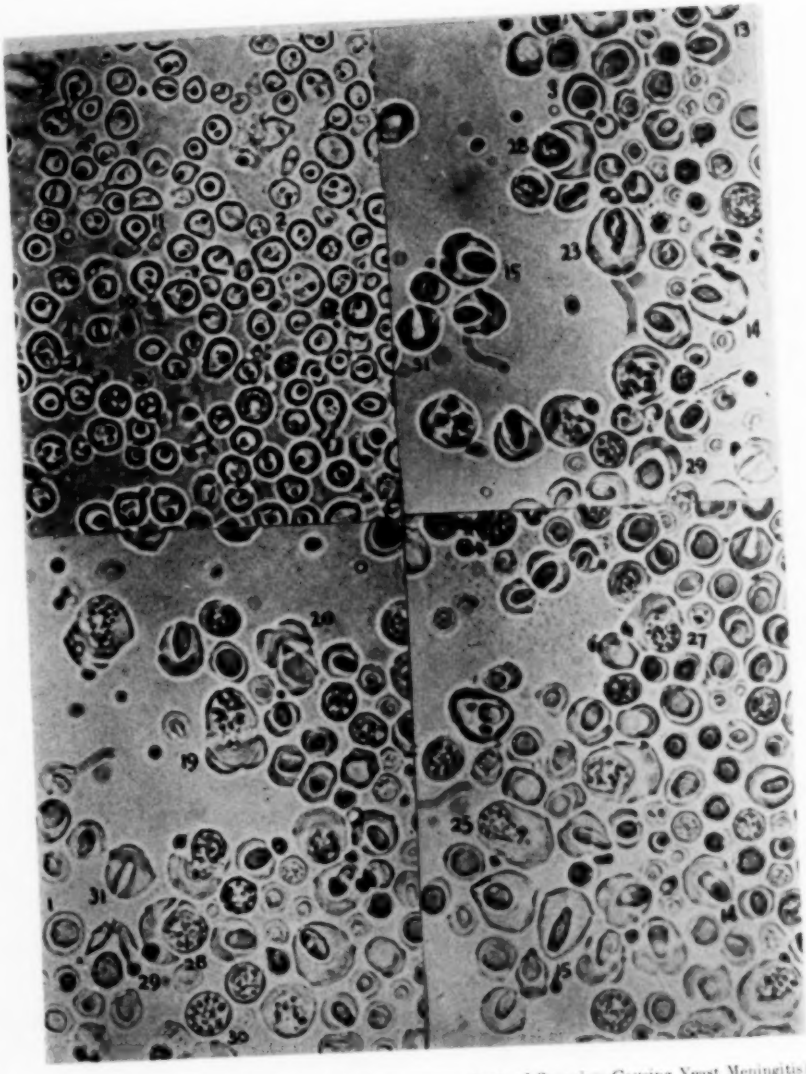
BENHAM, R. W. 1934 Arch. Dermat and Syph., **30**, 385–400.
BENHAM, R. W. 1935a Mycologia, **27**, 496–502.

BENHAM, R. W. 1935b Jour. Infect. Dis., **57**, 255–274.
BUSCHKE, A. 1898 Deutsch. Derm. Gesell., Verhandl., **5**, 181–222.
BUSSE, O. 1894 Centralbl. f. Bakt., **16**, 175–180.
CASTELLANI, A. 1935 Jour. Trop. Med., **36**, 297–322.
DODGE, C. W. 1935 Medical Mycology. C. V. Mosby Company, St. Louis.
FIROR, W. M. 1926 Internat. S. Digest, **2**, 3–8.
FITCHETT, M. S., AND WEIDMAN, F. W. 1934 Arch. Path., **18**, 225–244.
FREEMAN, W., AND WEIDMAN, F. W. 1923 Arch. Neurol. and Psychiat., **9**, 589–603.
KLOECKER 1909 Compt. rend. d. trav. du lab. Carlsberg, **7**, 273.
LODDER, J. 1934 Die Anaskosporogenen Hefen. Amsterdam. Centraalbureau voor Schimmelcultures.
OTA, M. 1923 Ann. Parasitol. Hum. Comp., **1**, 128–134.
OTA, M. 1924a Derm. Woch., **78**, 284–316.
OTA, M. 1924b Ann. Parasitol. Hum. Comp., **2**, 35–37.
PERSOON, C. H. 1801 Synopsis methodica fungorum. Goettingae.
RAPPAPORT, B. Z., AND KAPLAN, B. 1926 Arch. Path. and Lab. Med., **1**, 720–741.
SHAPIRO, L. L., AND NEAL, J. B. 1925 Arch. Neurol. and Psychiat., **13**, 174–190.
SHEPPE, W. M. 1924 Amer. Jour. Med. Sci., **167**, 91–108.
STODDARD, J. L., AND CUTLER, E. C. 1916 Monog. Rockefeller Inst. Med. Res., **6**, 1–98.

PLATE 1

PHOTOMICROGRAPH, APPROXIMATELY × 625

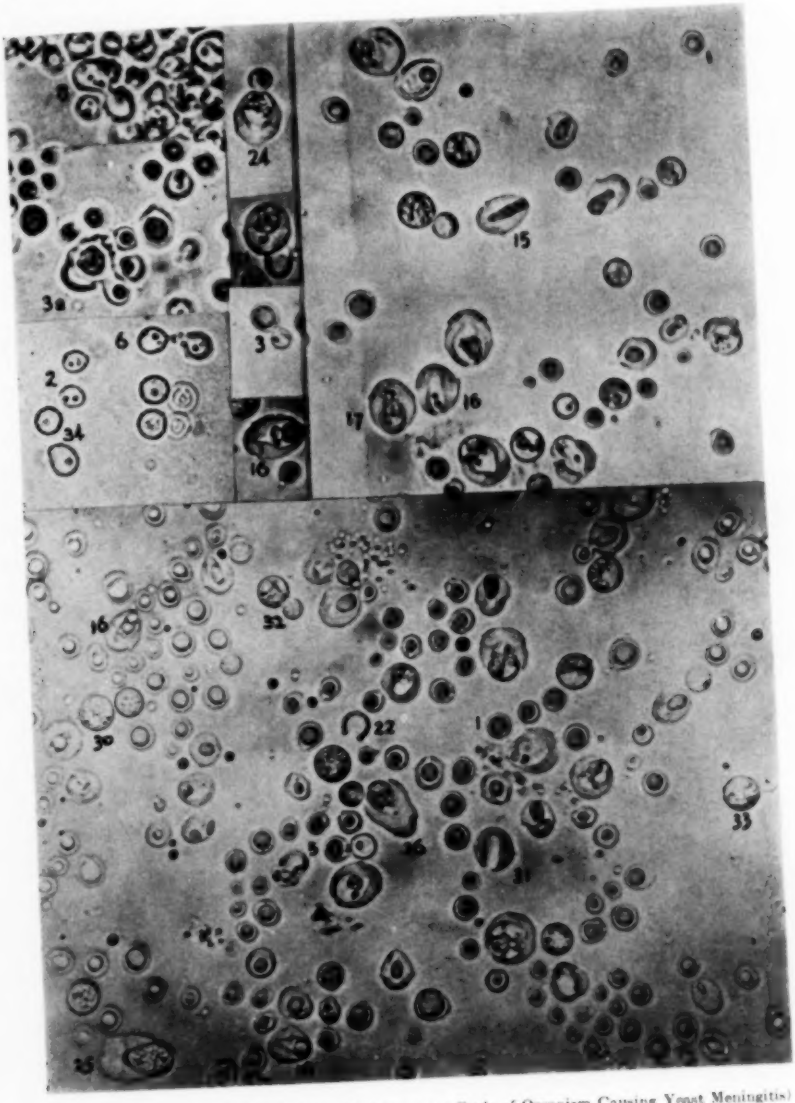
(Numbers designating cells correspond with numbers in plate III)



(Ramona L. Todd and Walter W. Herrmann: Cycle of Organism Causing Yeast Meningitis)

PLATE 2

PHOTOMICROGRAPH, APPROXIMATELY × 425
(Numbers designating cells correspond with numbers in plate III)



(Ramona L. Todd and Walter W. Herrmann: Cycle of Organism Causing Yeast Meningitis)

PLATE 3

CELLS FROM PHOTOMICROGRAPHS ARRANGED IN ORDER OF THEIR DEVELOPMENT, THUS REPRESENTING THE LIFE CYCLE OF THE ORGANISM

Magnification of each cell, approximately × 500

(Ramona L. Todd and Walter W. Herrmann: Cycle of Organism Causing Yeast Meningitis)

A STUDY OF VARIATION IN HEMOLYTIC STREPTOCOCCI FROM SCARLET FEVER AND ERYSIPELAS

II. COMPARATIVE VIRULENCE, CARBOHYDRATE FERMENTATION, TOXIN PRODUCTION OF THE S AND R STRAINS. PROTECTIVE POWER OF S AND R VACCINES. REVERSION

SOPHIE SPICER

ASSISTED BY EMILY L. BLOOM AND MARY F. GONSHOREK

Bureau of Laboratories, Department of Health, New York City

Received for publication March 24, 1936

In a previous article (Spicer, 1933) I reported the results of a study concerning variation of seven strains of hemolytic streptococci from scarlet fever and erysipelas. This study embraced the morphological, cultural and some serological phases in the dissociative processes both spontaneous and induced. The strains were type strains representative of four of our scarlet fever groups and three of our erysipelas groups and were designated, for the sake of clarity, as type I, type sub I, type III and type IV of scarlet fever, and type I, type II and type sub II of erysipelas, corresponding to the types represented by the strains in question. The rough variants obtained from each were designated as R variants of their particular type strains.

I now wish to report the results of experiments made to determine the comparative virulence, carbohydrate fermentation reactions, toxin production and protective power of vaccines made from the rough and the smooth variants of the same seven strains of streptococci. Reversion of the rough variants to the smooth type will also be considered.

VIRULENCE OF R STRAINS AS COMPARED WITH S STRAINS

As stated in the preliminary paper, the strains worked with were old stock strains, and as such were of low virulence. It

was therefore difficult to determine the comparative virulence of the R variants with that of the original strains. An attempt was made to raise the virulence of each of the S and R strains by mouse passage.

While the virulence of most of the S strains could be raised by continuous mouse passage so that 10^{-6} and 10^{-7} cc. of culture killed a mouse in from twenty-four to seventy-two hours, the R strains did not yield such results. They could not be carried from mouse to mouse and it usually required as much as 1 cc. of a heavy eighteen-hour culture to kill the first mouse in from seventy-two to ninety-six hours.

An attempt was also made to induce variation from the virulent S-passed mouse cultures and then test the subsequent S and R variants. The method used to incite the process was the same as that in obtaining the other variants, i.e., by aging. The results, however, were not so successful as those obtained from the original strains. The new variants were not of the extreme rough type, but of the coarse variety. Comparative tests were made with these variants.

It was found that while both were virulent to a certain extent, it was always the S form that proved to be more virulent for white mice, except in one case, that of type sub II erysipelas, in which both the S and the R were of equal virulence.

A new R variant of one of the scarlet fever type strains was recently found, that of type IV. This strain on passage through mice, yielded an R variant of equal virulence with that of the original S strain from which it was obtained.

CARBOHYDRATE FERMENTATION REACTION

Tests on carbohydrate fermentation were carried out with all the S and R strains under study. The fermentation tests were made in sugar-free veal-infusion agar slants to which had been added horse serum, Andrade indicator, and one of the sugars to be tested (lactose, mannitol, raffinose, salicin and inulin). Each tube was inoculated, butt and slant, with a fresh culture of S or R strain and incubated for eight days. Positive and negative controls of each sugar were run. Readings were made daily.

Results. All the rough variants showed the same fermentative power as the homologous S or parent strains, except in one case, that of type II erysipelas in which a *subacidus*, by Holman's classification, changed on dissociation to a *pyogenes*, thus showing a new physiological characteristic in its assumed ability to ferment lactose and salicin, although it did so only slowly.

TOXIN PRODUCTION

The rough and the smooth strains were planted in veal broth and incubated for six hours. Each strain was then planted in trypsin digest broth and incubated for four days. They were streaked daily on blood agar plates and examined for any possible changes.

All the rough strains, and to a lesser degree the smooth strains, showed dissociative changes during the four days' growth in the trypsin digest broth and, moreover, the ratio of the different variants in the same culture bottle changed from day to day.

The filtrates from the cultures of the S and R strains thus grown for four days in trypsin digest broth were either found to be alike in toxicity or the rough was somewhat weaker than the smooth, as tested on rabbit's skin. However, no definite conclusions as to the comparative toxin production by either strain can be drawn, since a thorough study of this phase of bacterial variation would involve the stabilization of both type strains in the special media used. This is time-consuming and not always successful. So much however may be said with certainty: If a culture consists of a mixture of variants, there is no way of telling which is the better toxigenic strain, especially when the ratio of the R to the S varies from day to day, as I have found it to do on daily examination of the cultures.

COMPARATIVE PROTECTION AFFORDED TO MICE IMMUNIZED WITH VACCINES PREPARED FROM R AND S VARIANTS

Comparative protection tests were conducted with the S and R forms of three of the strains, those most stable and of the extreme rough type. These were: type I and type II erysipelas and type IV scarlet fever. Killed, formalinized vaccines were used

for immunization of the white mice and were prepared as follows: The S and R strains taken from stock cultures were first streaked on blood agar plates and examined for purity of type. They were then transplanted into beef-heart broth, incubated for six hours, planted on vitamin agar slants in Blake pint bottles and incubated for eighteen hours. The growth from the Blake bottles was then washed off with saline solution, the cells collected into centrifuge tubes and centrifugated for thirty minutes. The supernatant fluid was then poured off and the cells washed once and resuspended in formalinized saline solution (0.1 per cent formalin in 0.85 per cent sodium chloride solution). The vaccines were standardized by comparing the turbidity of the cell suspension with a set of Fuller's earth standard turbidity tubes. Both the S and the R vaccines were adjusted to the same turbidity standard and were equivalent to about three billion organisms per cubic centimeter.

White mice, ranging from 18 to 21 grams in weight, were used in the protection tests. The mice received three injections intraperitoneally on alternate days, first and second injections consisting of 0.5 cc. and the third injection of 1 cc. of vaccine. On the seventh day after the last injection the mice were tested for immunity with virulent S cultures. The method of culture administration was that detailed in a previous publication (Spicer, 1935).

The test strains were the homologous S of two of the three vaccine strains, those of type I erysipelas and type IV scarlet fever. The vaccines of type II erysipelas could not be tested against the homologous S as this strain is non-virulent. Instead, it was tested against the S or original strain of type I erysipelas.

The immunity afforded to the mice by the S and R vaccines was also tested for comparison against unrelated, heterologous strains of hemolytic streptococci. These included a strain from each of the following conditions: erysipelas, post-scarlet fever erysipelas, puerperal sepsis and general septicemia. All the test strains were rendered virulent by mouse passage so that less than ten organisms, or 10^{-7} to 10^{-8} cc., killed a mouse in from twenty-four to forty-eight hours. From previous experience we found that more uniform and more reliable results could be

obtained in protection tests when the virulence of the test strain was enhanced to its maximum.

The results of the protection tests may be summarized as follows: Each rough variant strain when used as a vaccine produced an immunity in white mice equal to that produced by the smooth parent strain against the virulent homologous smooth culture.

The protection afforded by the R vaccines against virulent heterologous strains was much greater than that produced by the S strains in two out of three cases under experimentation. In one case the S protected somewhat better than the R against unrelated strains.

The rough variant of type II erysipelas gave the highest protection of the three R strains worked with, and moreover, it protected against heterologous strains to a higher degree than any of the others.

REVERSION OF ROUGH VARIANTS TO THE SMOOTH FORM

The study of reversion was made from the standpoint of morphology, sedimentation in broth cultures and saline suspensions on standing, agglutinin absorption reaction, and carbohydrate fermentation. Both induced and spontaneous reversion were considered.

I. Induced reversion. A number of reagents and factors were employed in an attempt to induce reversion of the R to the S forms, such as: (1) Growing the strains in beef-heart broth to which lithium chloride was added (0.05, 0.1 and 0.25 per cent); (2) growing the strains in peptone broth (5 and 10 per cent of peptone); (3) growing them in beef-heart broth of a high pH (8.1 to 9); (4) growing the strains at a high temperature (41° to 42°C.). In no case was there reversion to the original S form. There were changes to less rough or coarse but not to the real smooth variety. Aging of the cultures, on the other hand, proved to be more successful.

Procedure. The rough strains, three in number, those of types I and II of erysipelas and type IV of scarlet fever, were chosen for this purpose because of their stability and extreme roughness. The other four rough variants spontaneously reverted to the

smooth type and will be considered later. Each strain was streaked on a blood agar plate and examined for purity. Plate cultures used for inoculation looked typically rough. The cell morphology of the R variants was alike in that they all exhibited very long chains of large cocci. One-hundred-cubic centimeter bottles of beef-heart broth (pH 7.4) were inoculated with the pure cultures from plates. Two bottles were inoculated with each strain and incubated at 37°C. One set of bottles was opened daily for streaking and the other was kept in the incubator to be streaked before discontinuing. No marked change was noticed before fifteen days of incubation. The observations were discontinued at the end of nineteen days.

Results. Erysipelas type I R grown for nineteen days in beef-heart broth yielded a smooth strain with the following characteristics:

Colony morphology: Finely granular, light, similar to the S original, but with tendency to ring formation after forty-eight hours' incubation.

Cell morphology: Short chains of small cocci, an occasional medium-sized chain of larger cocci.

Sedimentation in broth culture and saline suspension on standing: Somewhat granular supernatant, granular precipitate.

Agglutinin absorption reaction: Partially absorbs (about 50 per cent) from the original S.

Carbohydrate fermentation: Reacts the same as the original S and the R variant from which it reverted.

Erysipelas type II R grown for seventeen days in beef-heart broth yielded a smooth type as follows:

Colony morphology: Smooth, light. Lighter, smoother and somewhat smaller colony than the original S.

Cell morphology: Short chains of small cocci, an occasional long chain of large cocci.

Sedimentation: Clear supernatant, fine precipitate.

Agglutinin absorption: Partial absorbtion (about 50 per cent) from the original S.

Carbohydrate fermentation: Same as the R type from which it reverted, and not like the original S.

Scarlet fever type IV R variant, on growing for fifteen days in beef-heart broth changed as follows:

Colony morphology: Smooth, light, darker center, very much like but not exactly like the original S.

Cell morphology: Short chains of medium-sized cocci. Cells somewhat larger than those of the original S type.

Sedimentation: Slightly cloudy supernatant. Flaky precipitate which breaks up into fine suspension on shaking.

Agglutinin absorption: Partially absorbs (about 50 per cent) from the original S.

Carbohydrate fermentation: The reaction is the same as that of the S and the R.

II. Spontaneous reversion. The rough variants which spontaneously reverted to the smooth form were those of types I, sub I, and III of scarlet fever, and type sub II of erysipelas. Following is a description of the changes which these strains underwent on turning to the smooth form:

Scarlet fever type I R changed by successive stages to a smooth form.

Colony morphology: Smooth, light, finely granular, indistinguishable from the original S.

Cell morphology: Short chains of small cocci, occasional pairs of very large cocci.

Sedimentation: Cloudy, slightly granular supernatant, fine precipitate.

Agglutinin absorption: About 75 per cent absorption from the original S, almost but not complete absorption from the R from which it "reverted."

Carbohydrate fermentation: No change, same as the homologous S and R strains.

Scarlet fever type sub I R changed also by successive stages to a smooth with the following characteristics:

Colony morphology: Light, finely granular, but not quite so smooth as the original S form.

Cell morphology: Medium-sized chains of small cocci. Occasional chains of larger cocci.

Sedimentation: Cloudy supernatant, granular precipitate.

Agglutinin absorption: Absorbs agglutinins from the original S and R variant almost completely, leaving a very small fraction in each case.

Carbohydrate fermentation: No change, same as the original S and the R from which it "reverted."

Scarlet fever type III R variants, of which there were fourteen, very early reverted to the original S and proved to be identical in every respect, so far as tested, with the original S form.

Erysipelas type sub II R on reversion to the S showed the following:

Colony morphology: Flat, smooth, somewhat granular border. Identical with the original S.

Cell morphology: Very short chains of small cocci.

Sedimentation: Almost clear supernatant, floccular precipitate which breaks up into a coarsely granular suspension on shaking.

Agglutinin absorption: Completely absorbs from the original S and the R. (The S and R completely cross-absorb.)

Carbohydrate fermentation: No change, same as the S and the R.

DISCUSSION

The results here recorded, of various tests employed in the study of dissociative changes in hemolytic streptococci, confirm the findings previously reported (Spicer, 1933) in that a change in colony morphology does not necessarily indicate a change in other characteristics. On the other hand, a strain may change in several characteristics with the colony morphology altering very slightly. It might be advantageous to keep these facts in mind, especially for those workers who are engaged in the preparation of vaccines and toxins. The realization of the possibility of such changes may at times help to elucidate some otherwise unexplainable occurrences relative to the changeability in the potency of such products.

The type that carries the virulence is generally considered the "normal" or "typical" form of an organism. In all other pathogenic bacterial species so far studied, outside of the streptococcus, it seems that the S form (with very few exceptions) is the "normal" and the one with which virulence is associated. No gener-

alization of this sort can be made about the streptococcal group. Cowan (1922), who was the first to demonstrate dissociation of hemolytic streptococci into rough and smooth types, found the S form much more virulent than the R. Andrewes' (1928) findings regarding virulence of S and R types of hemolytic streptococci were exactly opposite to those of Cowan. Todd (1928) obtained rough and smooth forms of hemolytic streptococci. Examination of these two types led him to the unexpected observation that the S types were permanently attenuated and formed clumps in broth whereas the R forms were virulent and produced uniform turbidity in broth. To avoid confusion which might arise from this unusual relationship between virulence and colony form he designated the virulent type as "matt" and the avirulent as "glossy." Eagles (1928) in his study of two strains of hemolytic streptococci found enhanced virulence in the R variant of one and lack of virulence in that of the other, and concluded that colony morphology is not a reliable index of virulence. In the present study, the strains worked with were old stock strains and as such were much attenuated. The virulence of most of the S strains could be raised by mouse passage to quite a high degree. The R strains obtained from these S, on the other hand, were all avirulent and could not be raised in virulence by mouse passage. When enforced variation was applied to the S strains which were rendered virulent by mouse passage it was found that the resultant R variants were also virulent although to a lesser degree than the parent S in all but one case, that in which the S and R were of equal virulence. One other R variant, which was obtained from an S on mouse passage, proved to be of equal virulence with the S type.

The R variants gave better protection to mice when used as vaccines against virulent S strains than the S variants. One R strain, in particular, though avirulent, gave very high protection against virulent, unrelated S strains. This is not in agreement with Day who maintains that the more virulent a strain is the better immunity it confers upon mice and rabbits when used as a vaccine, and that when the virulence of a strain is enhanced to its maximum and used as a vaccine it is capable of protecting against

other types of streptococci. The results of the protection tests in this study agree, however, with those of Cowan (1923) who found that the less virulent, R strains gave considerable protection to mice against virulent homologous and heterologous S types.

Hadley (1927), considering the subject of reversion, suggests that the R to S transformation may take place not through true reversion but through further progression and may thus effect, what he terms, an antigenic cycle, rather than a back-and-forth variation. The results of the agglutinin absorption tests of the "normal" S types and the "reverted" S seem to point to the existence of such a cycle. The fact that "spontaneous" reversion, or reversion taking place without enforcement, by gradual stages, finally yielded smooths which were, according to the tests, antigenically alike, or nearly so, with the original S types strengthens this contention. In the induced reversion the resultant S types showed only partial return to the S form, judging from the partial absorption of the agglutinins from the "normal" S serum by the "reverted" strains. It is possible that the method for enforcement used was not conducive to a complete return to the original S type. It may also be noted from the results of this study of reversion that the return to the S (colony texture) does not indicate in each case the return of other characteristics, and that true return to the "normal" S form is more the result of spontaneous than enforced reversion.

REFERENCES

ANDREWES, F. W. 1928 Jour. Path. and Bact., **31**, 132.
COWAN, MARY 1922 Brit. Jour. Exper. Path., **3**, 187.
COWAN, MARY 1923 Brit. Jour. Exper. Path., **4**, 241.
DAY, H. B. 1933 Jour. Path. and Bact., **37**, 169.
EAGLES, G. H. 1928 Brit. Jour. Exper. Path., **9**, 330.
HADLEY, P. 1927 Jour. Inf. Dis., **40**, 1.
SPICER, SOPHIE 1933 Jour. Bact., **26**, 505.
SPICER, SOPHIE 1935 Jour. Immun., **28**, 399.
TODD, E. W. 1928 Brit. Jour. Exper. Path., **9**, 1.
TODD, E. W. 1928 Jour. Exper. Med., **48**, 493.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTH CENTRAL BRANCH

THIRD SEMI-ANNUAL MEETING, IOWA STATE COLLEGE, MAY 1 AND 2, 1936

THE USE OF THE HIGH-RATE TRICKLING FILTER IN THE TREATMENT OF CREAMERY WASTE. *H. O. Halvorson*, Department of Bacteriology, University of Minnesota.

Studies have been made on the use of a high-rate trickling filter in the treatment of creamery waste. It has been found that strong creamery waste can be pre-treated to reduce its strength to that of city sewage by passing it through a trickling filter at the rate of from 20 to 25 million gallons per acre per day. For very strong waste, it has been found desirable to make use of a storage tank in which souring is allowed to occur and in which the casein precipitates. The liquid pumped from this tank at a pH of 4.5 is mixed with a portion of effluent from the filter, and the mixture is then pumped through the filter. The effluent from the filter has a pH in excess of 7. Best results are obtained if, instead of rock, the filter is composed of a specially fabricated tile which is so placed in the filter as to form a series of vertical conduits 1 inch in diameter. The bacteria cling to the vertical walls as readily as to rock. The use of such tile overcomes channeling and gives a larger amount of surface per cubic foot than does the average sized rock used in trickling filters. The use of tile also cuts down danger of ponding.

THE USE OF RASCHIG RINGS AS A TRICKLING FILTER MEDIUM. *Max Levine, Ralph Luebbers, W. E. Galligan and Reese Vaughn*, Engineering Experiment Station, Iowa State College, Ames, Iowa.

A settled domestic sewage with a B.O.D. of 186 p.p.m. to 226 p.p.m. was effectively purified at rates up to 16,000,000 g.p.a.p.d. by trickling filters and settling. A specially designed perforated clay block, 1½ to 3-inch quartzite and 1-inch hollow clay cylinders, known as Raschig rings, were employed as filter media. The reduction of B.O.D. by filtration and settling was 78.6, 81.8 and 89.6 per cent on the quartzite, the perforated clay block and the Raschig rings, respectively.

Nitrification was distinctly better with the Raschig rings than with the other filling materials, 3.5 p.p.m. of nitrates being produced at the 16,000,000 gallon rate.

Filtration at 2,000,000 g.p.a.p.d. followed by settling the effluent resulted in the removal of 375 pounds of B.O.D. per acre-foot of filter when quartzite was the filter medium, and 487 pounds B.O.D. per acre-foot with the 1 inch Raschig rings. At the 16,000,000 gallon rate of filtration the corresponding removals of B.O.D. constituents were 3,950 pounds for quartzite and 4,500 pounds for Raschig rings per acre-foot of filter.

The biological efficiency of trickling filters for the removal of oxygen-demanding constituents has not been fully utilized in standard practice.

STUDIES ON THE GERMICIDAL PROPERTIES OF AZOCHLORAMID. *E. J. Ordal*, Department of Bacteriology, University of Minnesota.

The object of this study was to determine the effect of the addition of soaps and wetting agents to buffer solutions which contained N-N-dichloroazodicarbonamidine, a compound commonly called Azochloramid.

Soaps or wetting agents such as sodium oleate, sodium resinate, sodium laurate, sodium lauryl sulfate, sodium ricinoleate, etc., were added to buffer solutions which contained Azochloramid. The buffer solutions ranged from pH 8 to pH 12.3 and were made from sodium bicarbonate, sodium carbonate, and sodium metasilicate.

The germicidal value of the various mixtures was determined by a modified F.D.A. method using a 24-hour culture of *Staphylococcus aureus* at 37°C.

The addition of a soap or wetting agent to a mixture of a buffer salt and Azochloramid definitely increased the germicidal value of the solution. This effect varied with the pH of the solution and with the particular soap or wetting agent used. For example, the addition of sodium oleate or of sodium lauryl sulfate gave better results than the addition of sodium laurate.

Consequently, Azochloramid may be added to mixtures of alkali salts and soaps or wetting agents to produce germicidal soap mixtures.

A COMPARISON OF THE PROPERTIES OF CLOSTRIDIUM BUTYRICUM PRAZMOWSKI AND BACILLUS SACCHAROBUTYRICUS VON KLECKI. *Newell O. Sjolander and Elizabeth McCoy*, University of Wisconsin.

QUANTITATIVE ASPECTS OF A DIRECT MICROSCOPIC METHOD FOR STUDYING DISTRIBUTION OF BACTERIA IN WATER. *Arthur T. Henrici*, University of Minnesota.

STUDIES ON THE MECHANISM OF NITROGEN FIXATION BY LEGUMINOSAE. THE pO_2 FUNCTION. *P. W. Wilson and V. S. Bond.* University of Wisconsin.

The effect of the pO_2 in the atmosphere on the assimilation of free and combined nitrogen by red clover was investigated over the range of pO_2 from 0.01 to 0.60 atm. In the absence of an added inert gas, i.e., only N_2, O_2, and CO_2 present, the assimilation of both free and combined nitrogen is independent of pO_2 (within experimental error) between partial pressures of oxygen from 0.05 to 0.40 atm.; below 0.05 and above 0.40 atm. the assimilation of both free and combined nitrogen decreases. Since the pO_2 function is independent of the source of nitrogen under the conditions specified, molecular oxygen as such does not appear to be directly concerned in symbiotic nitrogen fixation process.

Commercial electrolytic hydrogen contains a substance which apparently exerts a specific inhibitory effect on the fixation process. In the presence of this source of hydrogen the pO_2 function of plants fixing nitrogen differs from that of plants assimilating combined nitrogen. In this case the break in the curve for *plants using elemental nitrogen* is raised from 0.05 to 0.10 atm. indicative that an intimate relationship may exist between the enzyme system responsible for fixation of free nitrogen and the oxidation system present.

ON THE USE OF THE RATIO METHOD FOR DIRECT MICROSCOPIC COUNTING OF SOIL ORGANISMS. *R. H. Walker and Albert Klingebiel*, Iowa State College.

THE OCCURRENCE OF CAULOBACTERIALES IN SOIL. *C. E. Skinner and Victor Gitelman*, Department of Bacteriology, University of Minnesota.

Samples of Minnesota soil were inoculated into flasks of the following sterile media: tap water, tap water + 0.1 per cent nutrient broth, and tap water + 0.5 per cent broth. A slide was immersed in each flask before sterilization. After room temperature incubation for a few weeks, the slides were stained by crystal violet or Congo red. Many morphological types developed on the slides and in about one-half of the flasks of tap water and the 0.1 per cent broth, species of the order *Caulobacteriales* were found. Several samples of Florida soils were studied in the same way but few stalked bacteria were encountered.

SOME PHYSIOLOGICAL EFFECTS OF ACCESSORY GROWTH SUBSTANCES UPON RHIZOBIA. *D. W. Thorne and R. H. Walker*, Iowa State College.

A study of the root nodule bacteria showed that *Rhizobium meliloti*, *Rhizobium trifolii*, *Rhizobium leguminosarum* and *Rhizobium japonicum* were able to maintain growth when continuously cultured in mineral salts sucrose c.p. media with NH_4Cl or asparagin, but were unable to make any appreciable growth with KNO_3 as the nitrogen source. The inability to obtain growth of rhizobia in the KNO_3-sucrose c.p. medium employed in previous investigations may be accounted for by a deficiency in iron or an unfavorable potential.

The mean respiratory quotient of 5 species of *Rhizobium* for 24 hours was significantly lower in glucose media containing yeast extract than in glucose media containing either $NaNO_3$ or NH_4Cl. Brown cane sugar, soil extract, *Azotobacter vinelandii* medium extract and cysteine added to KNO_3 or NH_4Cl sucrose c.p. media brought about a decrease in the respiratory quotient of *Rhizobium trifolii* for the first few hours after inoculation. Yeast and alfalfa extracts and agar lowered the respiratory quotient of the organisms throughout a large part of the active growth period.

One of the important functions of accessory growth substances for rhizobia seems to be the provision of an initial hydrogen donator. A readily available hydrogen donator for the nodule bacteria appears to benefit them by lowering the oxidation reduction potential and by supplying a readily available initial source of energy.

THE INFECTIVE ABILITY OF RHIZOBIA OF THE SOYBEAN AND COWPEA CROSS INOCULATION GROUPS. *J. J. Reid and I. L. Baldwin*, University of Wisconsin.

THE OCCURRENCE AND DISTRIBUTION OF AZOTOBACTER IN IOWA SOILS. *William P. Martin and R. H. Walker*, Iowa State College.

Three hundred and twenty-two random samples representing a wide range of Iowa soils were examined for *Azotobacter*. The spontaneous culture soil plaque and selective culture agar plate methods were used; results were similar in all tests. About 33.5 per cent were positive, indicating that some factor or group of factors is limiting distribution.

pH, calcium carbonate, available phosphate and carbonate-phosphate ratio were determined on all samples. Available phosphate and pH appeared to be related to the presence of *Azotobacter*, the limiting value of pH being

about 6, and of phosphate about 50 pounds per acre. But there was some evidence that pH influences *Azotobacter* indirectly by regulating available phosphate. Calcium carbonate may also indirectly regulate growth by controlling pH. No correlation was found between the occurrence of *Azotobacter* and the carbonate-phosphate ratio.

Many samples with a pH above 6 and more than 50 pounds per acre of available phosphate contained no *Azotobacter*. Therefore other factors may limit the growth of this organism. These must be determined and proper soil treatments applied before *Azotobacter* may live and fix nitrogen in all soils.

NITROGEN RELATIONSHIPS IN YEAST GROWTH. *E. C. Saudek, D. R. Colingsworth, and I. L. Baldwin*, University of Wisconsin.

AN ANALYTIC SCHEMA FOR EXPERIMENTS WITH THE BACTERIAL CELL AS CONTRASTED WITH THE METHODS OF RESTRICTED EMPIRICISM. *A. E. Stearn*, University of Missouri.

Since any discipline which has developed to such a state that it may legitimately be termed a science must transcend the observations upon which it is based, and not merely consist of a catalog of such observations, an attempt was made to indicate, though not exhaustively, how much of a science could be built around the behavior of the bacterial cell, starting with the proposed thesis that the non-specific chemical behavior of bacteria is fairly completely explained on the assumption that organisms consist, to the extent of this behavior, of aqueous systems of two classes of ampholytes, the one more acidic than the other. The points treated included electrophoretic behavior; staining behavior toward dyes, toward mordants and toward decolorizers; chemical stasis; certain aspects of stoichiometry; flocculation by dyes; and buffer action. These general behaviors should not be considered as in a separate category from specificities. In many cases ideas of specificity have preceded more detailed knowledge, and often fall before the accumulation of such knowledge. As illustration, it was shown from quantum mechanical considerations that any dipole properly placed in a reacting system could act as the prosthetic group of a catalyst for a bimolecular reaction such as proteolytic hydrolysis.

A QUANTITATIVE STUDY OF CHITINOVOROUS BACTERIA IN SOIL. *Faith Dravis and C. E. Skinner*, Department of Bacteriology, University of Minnesota.

Chitin-destroying microörganisms were counted by means of the dilution method and selective liquid media from 26 soils. The numbers varied in normal soils from 288 per gram taken from a dry sand bank along the Mississippi to over one million per gram from a fertile cultivated black loam. Few of these were molds, many actinomycetes. Of six alkali soils dried and stored for more than two years, three still had a small population of chitinovorous bacteria, all actinomycetes. Very few of the bacteria picked at random from colonies which developed on soil-extract agar plate dilutions in 14 days were able to dissolve chitin when inoculated into selective media. Of these nearly all were actinomycetes. Slightly less than one-half of the molds which developed on acid peptone-glucose agar plates from soil were able to dissolve chitin.

A Method for Eliminating False Positive Tests with the Formate-Ricinoleate Medium in Testing for Coli-Aerogenes Bacteria in Milk. *C. E. Skinner and O. Honaas*, Department of Bacteriology, University of Minnesota.

The coli-aerogenes bacteria in samples of raw milk were counted by the dilution method using brilliant green-bile and formate-ricinoleate broths and employing the tables of Halvorson and Ziegler. The positive tubes were confirmed on eosin-methylene blue agar plates. All tubes showing gas on the former media yielded *Escherichia coli* or *aerobacter aerogenes*, but about one-third of the samples of milk gave one or more positive presumptive tests in the latter medium which could not be confirmed. All these tubes were basic to phenol red, whereas all tubes which were confirmed were acid to this indicator. From the tubes giving a false presumptive test, glucose-fermenting, lactose-non-fermenting aerobes were isolated. Each of these could be identified as a *Proteus* or a *Salmonella*. Allowing for the false presumptive tests, the formate-ricinoleate medium still gave a higher average count than the brilliant green-bile broth, but the difference was not significant since only some 50 samples with 5 tubes of each of three dilutions were used for each medium. The incorporation of phenol red in the formate-ricinoleate medium permits the elimination or the detection, in the preliminary enrichment medium, of aerobes unable to form gas from lactose, but forming gas from sodium formate or glucose.

Cell Inclusions in Neisseria. *Beryl B. Green and Charles Drake*, Department of Bacteriology, University of Minnesota.

A strain of *Neisseria perflava* was isolated from a normal throat, and has been grown on veal infusion agar. Within 48 hours after isolation it showed large vacuolated cells, and the proportion of such cells has increased. After 90 hours staining with Giemsa, eosinophilic granules ranging from small ones to large ones that almost fill the cell show up in many cells. Sometimes two or three granules are present in one cell. The bodies have much the appearance and staining qualities of the intranuclear inclusions produced in animal cells by the presence of a filterable virus. In view of the general acceptance of the bacteriophage as a filterable virus infecting bacteria, it would not seem unreasonable to postulate the existence of filterable viruses which infect bacteria with the production of intrabacterial bodies analogous to inclusion bodies in animal cells. We have, however, not yet been able to prepare a filtrate of the original culture which will infect other cultures of bacteria. Work on this phase of the problem is being continued. The colonies are rough, but no areas of lysis have been seen.

Types of Fox Encephalitis Virus. *R. G. Green*, Department of Bacteriology and Immunology, University of Minnesota.

The original description of the fox encephalitis virus included a virulence capable of producing an 80 per cent mortality under experimental conditions and a ranch mortality of 30 per cent. In groups of foxes passively immunized by a serum made from this virus, a mild type of the disease has been observed to develop showing a mortality of about one per cent. Under experimental conditions this virus produces a mortality of only 20 per cent, but with endothelial cell in-

clusions typical of encephalitis. Subsequent observations have indicated that this new type of the virus is immunologically distinct from the original. The original virulent virus is now referred to as "type A." The newly recognized virus of low virulence is described as "type B" fox encephalitis virus.

PURIFICATION OF FOX ENCEPHALITIS VIRUS BY TRYPTIC DIGESTION. *R. G. Green*, Department of Bacteriology and Immunology, University of Minnesota.

Smears made from the ependyma of foxes inoculated by cisterna puncture were stained to show the presence of the intranuclear inclusions of fox encephalitis. A suspension of ependymal scrapings containing inclusion bodies was digested by the use of trypsin and enterokinase. Definite clearing of the suspension was noted and cellular material could not be demonstrated by stained smears after digestion. Upon centrifuging, a sediment was obtained which was resuspended in distilled water. Ten similar washings were performed, using 10 cc. of liquid for each washing. Finally, suspended in saline solution, the sediment produced typical fox encephalitis when injected into foxes by cisterna puncture, although a low concentration of the virus was indicated. This procedure demonstrates the resistance of the virus to tryptic digestion and its occurrence under these conditions in fairly large masses capable of ordinary centrifugation.

FURTHER ATTEMPTS TO PRODUCE POLIOMYELITIS BY THE GASTRO-INTESTINAL ROUTE. *William S. Preston and Paul F. Clark*, Department of Bacteriology, University of Wisconsin.

Feces were collected throughout the prodromal and acute stages of the disease from three monkeys infected with Aycock virus and five with Rockefeller virus. The dialyzed fecal concentrate from the Aycock group was injected into two animals, that from the Rockefeller group into five. All animals remained well.

Two monkeys were injected according to Toomey's procedure, 70 cc. 10 per cent-cord-suspension into 15 cm. of the ileum enclosed between clamps. After ten minutes the clamps were removed and the wound closed. The animals remained well.

According to Lennette and Hudson, virus given intravenously is excreted into the nasopharynx and intravenous injections are ineffective following section of the olfactory tract. This, together with the finding of Schultz and Gebhardt that acid phosphate washing increases the percentage of infections by the nasal route, led us to combine nasal washing with gastric feeding.

The nasal cavities of three monkeys were irrigated with 25 cc. acid-phosphate buffer, daily for five days. On the second, third, and fourth of these days, the washing was followed by 25 cc. of 10 per cent virus into the stomach by tube. One of the animals died of dysentery and the others remained well.

We conclude that the gastro-intestinal tract is a negligible factor in the spread of poliomyelitis.

THE EFFECT OF ARTIFICIAL FEVER ON THE DEVELOPMENT OF ANTIBODIES. *Harold V. Ellingson and Paul F. Clark*, Department of Bacteriology, Medical School, University of Wisconsin, Madison.

Using an electrically-heated box, artificial fevers of 40°C. were induced in rabbits for three-hour periods daily

during immunization with sheep red-blood corpuscles.

Three series of twelve rabbits were used, there being six test animals and six controls per group. In all cases, erythrocyte counts and chloride determinations of the whole blood, made before and after fevers, indicated a temporary concentration of the blood, lasting no longer than twenty-four hours. Complement either was not affected, or was only slightly depressed.

In one series, mild fevers of 40°C. induced daily for six days in the test animals seemed to have no significant influence on the development of antibodies. In the other two series, test animals which had been given more severe fevers of 42° showed lower concentrations of circulating antibodies than did control animals of the same groups.

THE FATE OF YEASTS IN THE INOCULATED ANIMAL. *Cora Rust Owen*, Department of Bacteriology, University of Minnesota.

The constant lack of leucocytic infiltration in disease lesions caused by true yeasts and related organisms suggested that a study be made of the mechanisms by which the animal body eliminates such parasites. Four yeasts, one pathogenic for man and guinea pigs, and four monilias, one pathogenic for rabbits, were used in these experiments. Pathogens disappeared from the blood of rabbits much more slowly than non-pathogens. Immunization slightly reduced the time necessary for non-pathogens and gave irregular results with pathogens. Skin tests made with living cultures of the organisms showed that some degree of allergy developed from the injections, especially of pathogens. Sections of the organs showed abscesses in the spleen, kidneys, suprarenals, and brain of animals injected with the very pathogenic monilia. The organs of rabbits injected with non-pathogens showed no signs of yeasts, those injected with pathogens showed many degenerating yeasts in the macrophages of the spleen. Phagocytosis experiments on guinea pigs showed that pathogens disappeared from the peritoneal fluid much less rapidly than did non-pathogens and tended to form capsules. Sera from normal rabbits possessed no lethal power against yeasts; that from a rabbit hyperimmunized to *Torula histolytica* possessed such power only against that organism.

BUFFERED BLOOD CULTURES. *Casper I. Nelson*, N.D. Agricultural College.

The use of a buffer solution (McIlvaine's) composed of 1.83 cc. of a 0.1-molar citric acid solution combined with 18.17 cc. of a 0.2-molar disodium phosphate and having a reaction of pH 7.4 provides a medium for the culture of organisms in blood. Three to five cubic centimeters of blood drawn aseptically into 10 cc. of buffer solution lacks the inhibiting effect which often makes blood culturing ineffective. The blood does not clot. The blood serum constituents serve as nutrients.

Cultures have been easily obtained of various streptococci, *Brucella abortus*, *Eberthella typhosas*, and other organisms associated with fever as well as others of indeterminate function. The method has had successful use for bedside diagnosis.

"VITAMIN G" IN COCCIDIAN INFECTION. *Elery R. Becker and Neal F. Morehouse*, Iowa State College.

The intestinal location and life cycle of *Coccidia* make oocyst counts an index to their multiplication. Quantitative data were based on counts by the dilution method subsequent to infec-

tion with uniform numbers of oocysts in reference and test series. The host was the rat; the parasite, *Eimeria miyairii*; the reference diet, a basal, plus 10 per cent powdered yeast. Oocyst counts from hosts on a ration deficient in vitamins B and G were about a fifth of the normals; from G-deficient hosts, a third of normal; B-deficient hosts, equal to normal. Liver extract equivalent to 4 grams as a daily supplement to basal diet conditioned counts a third to fourth of normal; 2.0 per cent powdered liver and 2.5 per cent rice polish, counts a seventh of normal; 10.0 per cent powdered liver and 4.0 per cent rice polish, counts a third of normal. Only the last ration gave host growth equal to normal or reference series. Powdered skim milk as the sole source of vitamins B and G gave slight growth, but parasite counts were only a small fraction of normal. Liver and skim milk are either deficient in a coccidium accelerant or restrain their numerical increase by positive means.

ESSENTIAL GROWTH FACTORS FOR THE PROPIONIC ACID BACTERIA. *H. G. Wood, E. L. Tatum and W. H. Peterson*, Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin.

The propionic acid bacteria require small amounts of unknown substances for growth. An ether extract of acidified yeast extract (Difco) contains stimulants for these bacteria. The simplest medium on which the propionic acid bacteria have been grown is: ether extract of yeast extract (6.0 mgm. per 100 ml. of medium), ammonium sulphate 0.3 per cent, glucose 1.0 per cent, and Speakman's inorganic salts.

Tatum and Peterson have shown recently that potato contains a stimulant which is not precipitated by Neuberg's reagent or lead acetate and ammonia. A stimulant was also found in corn. These stimulants are ether-extractable acids. A combination of 1.0 mgm. of corn ether extract and 8.0 mgm. of potato ether extract per 100 ml. of medium is equivalent to the ether yeast extract. Only preliminary attempts have been made to purify the stimulants.

The stimulants from yeast are soluble in chloroform and benzene; insoluble in petroleum ether. The barium salt is soluble in 95 per cent alcohol but insoluble in acetone. None of the stimulants is extractable with ether as the sodium salts; they are all absorbed on norite and may be eluted. They give negative tests for amino acids with ninhydrin.

The addition of hydrolyzed casein to the $(NH_4)_2SO_4$ medium increases the growth.

ENZYMATIC RACEMIZATION OF DEXTRO LACTIC ACID. *E. L. Tatum and W. H. Peterson*, Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin.

It has been shown by Tatum, Peterson and Fred (Biochem. Jour., *26*, 846) that when certain lactic acid bacteria, which normally produce optically active lactic acid, are grown in association with *Clostridium acetobutylicum* inactive acid is formed. Butyric acid bacteria (*Clostridium butylicum*) have the same racemizing effect.

When dextro-lactic acid is added as the calcium salt to a fermenting culture of either the butyl alcohol or butyric acid organisms, it is partially fermented, and the remainder is changed to inactive acid.

This racemization appears to be brought about by enzymatic reactions.

If calcium d-lactate is added to 24-hour cultures of *Clostridium acetobutylicum* and *Clostridium butylicum* to which toluene and chloroform were also added, the lactic acid is racemized after incubation at 37°C. for 3 days. No acid is destroyed. If a 24-hour culture is heated at 68°C. for 10 minutes before adding the calcium d-lactate and antiseptics, racemization does not take place. This is further evidence of the enzymatic nature of the reaction.

Some evidence indicates that a coenzyme may be essential. Neither the cells nor the centrifuged medium racemize d-lactic acid, but a combination of the two is effective.

THE RÔLE OF PHOSPHOGLYCERIC ACID IN BACTERIAL DISSIMILATION. *R. W. Stone and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames, Iowa.

Phosphoglyceric acid, a key intermediate in the Embden-Meyerhof scheme of muscle and yeast glucolysis, has been isolated from various bacteria using fresh cell paste in a mixture of glucose, hexosediphosphate, phosphate buffer, sodium fluoride, acetaldehyde, $MgCl_2$, and toluene. The pure barium salt of phosphoglyceric acid has been obtained in relatively large quantities from *Aerobacter indologenes*, *Citrobacter freundii*, *Escherichia coli*, *Bacillus subtilis*, *Serratia marcescens*, and three species of *Propionibacterium*. Small amounts have been obtained from *Lactobacillus pentoaceticus*, *L. plantarum*, and *Azotobacter vinelandii*. *Clostridium butylicum* has yielded only a trace. The intermediate acid is also produced in the absence of acetaldehyde and very small amounts have been obtained in the absence of toluene. Aeration increases the yield, while under anaerobic conditions the yield is decreased. Aging the cell paste at 10°C. decreases the yield, although even dried bacteria will still give some phosphoglyceric acid. In view of its general occurrence it is suggested that phosphoglyceric acid plays a rôle in bacterial metabolism comparable to its function in yeast and muscle glucolysis. As methylglyoxal is at present generally regarded as the key intermediate in schemes of bacterial dissimilation, re-evaluation of the rôle of the glyoxal is necessitated by these findings.

DISSIMILATION OF GLUCOSE AND XYLOSE BY AEROBACTER INDOLOGENES. *Howard Reynolds and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames, Iowa.

Study of the dissimilation of glucose by serial analysis of fermentation liquors of *Aerobacter indologenes*, indicates that acetic acid accumulates in the early stages followed by a marked decrease. Graphical analysis shows a reciprocal relationship between acetic acid and 2,3-butyleneglycol + acetylmethyl-carbinol. The sum of the latter two + acetic acid calculated as the glycol was constant. Results indicate that acetic acid is a precursor of the glycol. Glucose media containing added acetate fermented by *A. indologenes* produced no hydrogen; about 40 per cent of the acetate disappeared, accompanied by an equivalent increase in 2,3-butyleneglycol. Studies yielding similar results were carried out with the Barcroft-Warburg respirometer and in test tubes. The ratio of CO_2:H_2 can be varied by the addition of acetate. In the test tube the ratio is a function of conditions governing the reduction of acetate. *Escherichia coli* does not show this behavior.

Products from glucose and xylose were qualitatively the same, excepting that succinic acid arose from the latter. Succinic acid appeared to be converted into 2,3-butyleneglycol, formic acid and CO_2.

NORTHERN CALIFORNIA-HAWAIIAN BRANCH

San Francisco, Feb. 26, 1936

PREVENTION OF INTRANASALLY INOCULATED POLIOMYELITIS IN MONKEYS BY INTRANASAL IRRIGATION WITH CHEMICAL AGENTS. *E. W. Schultz and L. P. Gebhardt*, Department of Bacteriology and Experimental Pathology, Stanford University, California.

Monkeys given intranasal irrigations with solutions of certain chemicals on three successive days have been found without further treatment to be resistant to repeated intranasal instillation of poliomyelitis virus administered 2 or more days after the chemical treatment. The results of several significant experiments indicate that intranasal instillations of picric acid (1 per cent) protects monkeys against intranasally instilled virus, for at least 2 days and sometimes as long as 90 days; mercurochrome (2 per cent) for periods as long as 60 days. More limited observations suggest that paranitrophenol (1 per cent), dinitrophenol (1 per cent), trinitrocresol (1 per cent), silver nitrate (1 per cent), protargol (5 per cent), erythrosine (2 per cent), eosin Y (1 per cent), neutral acriflavine (1 per cent), thionin (1 per cent), safranine (1 per cent), acid fuchsin (1 per cent), zinc sulphate (2 per cent), also afford protection against virus administered 2 days or more later, while ammonium picrate (1 per cent), argyrol (10 per cent) and congo red (1 per cent), uranine (1 per cent), are not protective under the same conditions. The actual duration of the protection offered by these and other promising agents are now being carefully studied. The effect of most of these agents does not seem to rest so much on virucidal action as on some sort of modification of the portal of entry, which renders it less permeable to the virus.

GROWTH OF ESCHERICHIA COLI IN A SMALL AMOUNT OF ORGANIC MATTER. *Victor Burke and Leslie Taschner*, State College of Washington, Pullman, Wash.

Six water-agar mediums were prepared by adding to washed agar; (1) river water filtered through filter paper, (2) tap water from a deep well but stored in a reservoir, (3) tap water filtered through a Berkefeld N filter, (4) freshly distilled water from a Stokes steam still, (5) tap water single-distilled with potassium permanganate and potassium hydroxide through a pyrex glass still, and (6) tap water double-distilled as in (5).

The cells from a 24-hour agar slant of *Escherichia coli* were washed three times in the double distilled water and then plated out on the six kinds of water agar. Visible growth occurred in two to four days on the first four kinds of water agar. Growth occurred about the fifth day in 30 out of 42 plates of the single-distilled water agar. No growth occurred within three weeks on the double-distilled water agar.

Comparable experiments using water without the agar and making plate counts to determine growth indicates that *E. coli* can multiply in filtered tap water and distilled water from a Stokes still. In 21 flasks of double-distilled water all the organisms were dead in 72 hours.

The double-distilled water appeared to be toxic. We have no evidence that bacteria die of starvation in 3 days. Growth occurred in such water upon the addition of a small amount of potato extract.

Graded amounts of potato extract were added to 31 flasks of double distilled water. The amount of organic

matter present as determined by the oxygen-consumed method and given as parts per million of oxygen consumed, ranged from 0.1124 to 0.5171. These flasks were inoculated with *E. coli*. Multiplication occurred in all flasks giving tests of 0.5087 p.p.m. and above of oxygen consumed. The tap water gave a test of 0.7352, water distilled through the Stokes still 0.6981, water double-distilled with potassium permanganate 0.0607.

CONNECTICUT VALLEY BRANCH

CONNECTICUT STATE COLLEGE, STORRS, CONNECTICUT, MAY 9, 1936

A CRITICAL STUDY OF THE FUSOBACTERIUM GENUS. I. BIOCHEMICAL AND SEROLOGICAL CLASSIFICATION. *E. H. Spaulding and L. F. Rettger*, Department of Bacteriology, Yale University, New Haven, Connecticut.

Eighty strains of Gram-negative, non-sporulating and non-motile anaerobes were isolated from a variety of human and animal sources. A biochemical study of these cultures revealed that, although members of a single genus, they comprise two groups with widely divergent properties. One group (I) is weakly saccharolytic and highly nitrogen-active, while the other (Group II) is strongly fermentative and relatively indifferent to the presence of certain nitrogen compounds. Each of the two groups can be further divided into two types on the basis of fermentation reactions.

The biochemical separation of Groups I and II is supported by agglutination and agglutinin-absorption tests, as in the division of Group II into two types. Group I, on the other hand, possesses a complex antigenic structure. A method is presented for the production of satisfactory agglutination antigens. The precipitin technique failed to yield reliable results; furthermore, a polysaccharide isolated by the method of Julianelle and Wieghard (1935) did not produce specific precipitation.

There was no apparent correlation between the sources of strains and the presence of types.

The results obtained from a study of morphological and cultural characteristics, growth requirements, pathogenicity and the phenomenon of variation will be presented in a subsequent report.

STUDIES ON ACID PRODUCTION BY THE COLI-AEROGENES GROUP. *Ralph L. France*, Department of Bacteriology and Physiology, Massachusetts State College, Amherst, Massachusetts.

In a medium containing 0.5 per cent proteose peptone, 0.5 per cent glucose, 0.3 per cent K_2HPO_4 and 0.1 per cent erythrosin, *Escherichia coli*, after forty-eight hours incubation, precipitated the dye, while *Aerobacter aerogenes* failed to do so. Intermediate strains varied in their reactions.

Daily determinations of pH were made on cultures grown in Clark and Lubs broth. Twelve *E. coli* strains reduced the pH to between 4.9 and 5.1 during twenty-four hours incubation. After forty-eight hours a further drop to 4.7 to 4.9 was observed. The reaction remained at this point throughout the final two days. Twelve *A. aerogenes* strains depressed the pH to between 5.25 and 5.70 by the end of the first twenty-four hours, after which partial reversion occurred. On the fourth day the pH was between 6.30 and 6.70.

Intermediate strains were divided into four groups:

Group I—Those giving reactions similar to *E. coli*.

Group II—Those giving reactions similar to *A. aerogenes*.

Group III—Those which reverted after the first day to give a final pH of 5.70 to 6.00.

Group IV—Those which depressed the pH to between 5.20 and 5.40 during the first twenty-four hours, and stayed within this range for the remaining three days.

OXIDATION-REDUCTION POTENTIALS OF CERTAIN ANAEROBIC AND FACULTATIVE ANAEROBIC BACTERIA. IV. EVIDENCE OF THE EXISTENCE OF POISING CAPACITY IN VARIOUS BACTERIOLOGICAL MEDIA. *Russell W. H. Gillespie*, Department of Bacteriology, Yale University, New Haven, Connecticut.

A phenomenon suggesting poising capacity has been observed in cultures of *Clostridium lentoputrescens*, intestinal lactobacilli, and a Gram-negative *Bacteroides* species. The trend of the potential-time curves of cultures of these relatively inactive bacteria makes it appear that the lag phase in such cultures extends over a period of days. In reality, a limited amount of growth apparently does occur during this period; the relatively static phase of potential can be attributed, therefore, to poising capacity exerted by some electromotively active constituent of the medium. Subsequent to the more or less complete reduction of this system (which, incidentally, appears to determine the anaerobic reduction potential of the sterile medium), as the result of bacterial activity, perhaps combined with "autoxidative" influence, a second phase of more active proliferation occurs.

The biological evidence summarized above is substantiated by chemical electro-titration. The correlation between the results obtained by the two methods is very nearly exact.

The demonstration of such capacity effect should, it seems, be a development of fundamental importance in the interpretation of bacterial O/R potentials.

OBSERVATIONS ON THE SEROLOGY OF STREPTOCOCCI OF BOVINE ORIGIN. *S. E. Hartsell and W. N. Plastridge*, Connecticut State College, Storrs, Connecticut.

The slide agglutination test and the precipitin test of Lancefield were used in examining 120 cultures that had been identified by the biochemical tests of Plastridge and his associates as either *Streptococcus mastitidis* group A (Lancefield's group B) or group B, or saprophytes.

In general, the three biochemical types were serologically distinct. Of seventy group A strains, sixty-five, which were associated with other evidence of mastitis, were divided into three serological types. Five group A cultures which failed to react with the three type sera were from samples from mastitis-free quarters. Of twenty-three group B cultures, fifteen reacted with one of three types of group B antiserum. Of seventeen cultures classed as saprophytes by biochemical tests, eleven reacted with one of three types of antiserum against saprophytic organisms, and six failed to react with any serum. Ten cultures which reduced methylene blue milk (1:5000), but otherwise resembled group A biochemically, were associated with other evidence of mastitis; they reacted with group A antisera. Members of group A which fermented salicin were identical serologically with those which

failed to do so. Strongly hemolytic sorbitol-fermenting cultures which reacted with our group A antisera were observed in one instance.

THE PREVALENCE AND CLASSIFICATION OF HEMOLYTIC STREPTOCOCCI IN PASTEURIZED MILK. *L. W. Slanetz*, Laboratory of Bacteriology, University of New Hampshire, Durham, New Hampshire.

Appreciable numbers of hemolytic streptococci have frequently been found in pasteurized milk. Samples of such milk with standard plate counts of 10,000 or less, sometimes contained as many as 50,000 hemolytic streptococci per milliliter. Meat-extract agar containing 1 per cent proteose peptone and 5 per cent sheep's blood proved to be very satisfactory for the detection and isolation of these bacteria.

Twenty cultures of these hemolytic streptococci were selected for detailed study. They were found to be extremely heat resistant, some strains withstanding a temperature of 70°C. for 30 minutes. When stained from liquid media, they occurred as long-chained streptococci. On blood agar, the colonies usually appeared as the so-called alpha-prime type.

Biochemical characteristics studied included fermentation reactions, final pH in glucose broth, hydrolysis of sodium hippurate, reduction of methylene blue, formation of soluble hemolysin, and digestion of human fibrin. Precipitin tests with group sera were also carried out.

Further studies are being conducted on the origin and identity of these hemolytic streptococci in pasteurized milk.

SEROLOGICAL TYPES OF SALMONELLA ISOLATED FROM PARATYPHOID IN CHICKS. *Erwin Jungherr and A. G. Borden*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

During the routine diagnosis of chick diseases, five cases of paratyphoid infection were observed in 1935 and 1936. No such cases were diagnosed during the preceding four years. Paratyphoid-like organisms were isolated from several chicks of each specimen lot and recognized as the etiological agents. Biochemical tests placed these organisms in the genus *Salmonella*. Strains determined to be smooth variants on the basis of morphology, colonial characteristics, boiling, salt-sensitivity, and Pampana tests were subjected to serological analysis, using the Kauffmann-White scheme for receptor analysis (Jour. Hyg., 1934, **34**: 333). Two of the organisms were found to resemble the one recently isolated by Jungherr and Wilcox (Jour. Inf. Dis., 1934, **55**: 390), and by Edwards (Jour. Bact., 1935, **30**: 365). These organisms belong to group B and are now known as *S. typhi-murium* var. Storrs. Two of the isolated organisms were shown to belong to group C, resembling *S. cholerae-suis* in the specific phase, and one strain was found to belong to group E, resembling *S. london*. The group C organisms differed from true suipestifer strains in fermenting arabinose, and the group E culture differed from *S. london* in failing to ferment inositol. The group E organism was able to absorb all antigenic factors from a known *S. london* serum and vice versa, but further tests are necessary to establish identity

THE ACTINOMYCES-LIKE NATURE OF A RECENTLY DESCRIBED "UNIDENTIFIED YELLOW PIGMENT-PRODUCING COCCUS." *Hazel B. Gillespie and Marion Bowen*, Department of Bac-

teriology, Yale University, New Haven, Connecticut.

In the September, 1935, issue of this Journal there appeared an article discussing the cyclic variation of an "unidentified yellow pigment-producing coccus." Using a sub-culture of the organism described, we have been able to reproduce practically all of the morphological changes reported. We cannot, however, accept the interpretation placed on these phenomena by the author.

Studying living preparations under oil immersion, we have observed the origin and manner of reproduction of the so-called cocci. Smeared out on extract agar blocks, they do not develop like cocci. They protrude as "sprouts" or "germination tubes" very much as do mold spores and the segmentation bodies of some of the actinomyces. The "germination tubes" elongate and later branch, forming delicate mycelial colonies. As colonies age the mycelium breaks up by repeated segmentation, producing coccus-like bodies, which appear in clusters because post-fission movement occurs. Although colonies, being of the mealy rather than of the leathery type, are easily removed from agar surfaces, part of the mycelium does penetrate deep into the medium. For these and other reasons Doctor Colien's "cocci" appear to us to be the final fragmentation forms of an Actinomyces-like organism. The cycle which he describes resembles very closely that reported in 1923 by Ørskov for certain group II Actinomyces.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

SOCIETY OF ILLINOIS BACTERIOLOGISTS

Annual Meeting, Chicago, May 1, 1936

Bacteriology as an Aid to the Milk Industry. *W. D. Dotterer*, The Bowman Dairy Co., Chicago.

Practical Applications of Fermentology in the Distilling Industry. *C. E. Boruff*, Hiram Walker and Sons, Inc., Peoria, Ill.

Experiences with Special Methods in the Isolation and Identification of Typhoid Bacilli from Blood, Feces and Urine. *H. J. Shaughnessy, N. Hall, J. O. Alberts and F. Friewer*, Division of Laboratories, Illinois Department of Public Health, Springfield, Illinois.

Blood cultures made by the usual methods on blood clots have yielded poor results. For the past three years cultures have been made in a medium described by Sellers (10 per cent ox-bile, 10 per cent glycerol, 2 per cent peptone) which dissolves the clot and inhibits contaminants. Of 2824 blood specimens examined, 505 showed Widal reactions in a dilution of 1:80 or higher and 144 gave positive clot cultures for typhoid or paratyphoid bacilli. Forty-seven positive cultures were obtained on specimens which gave negative Widals.

Most of the described methods for culturing feces and urine were tried without much success in these laboratories. Wilson and Blair bismuth brilliant-green sulphite medium has, how-

ever, yielded good results. Of 10,199 specimens examined, 179 were positive on both Wilson and Blair and Endo media, 262 were positive on Wilson and Blair only and 27 were positive on Endo only. Limited experience indicates that preliminary enrichment in Selinite F followed by plating on desoxycholate citrate agar will give results slightly superior to the Wilson and Blair medium.

All of the above results were based on thorough identification by morphologic, tinctorial, biochemical and serological tests. A chart for systematic study of these characteristics was presented.

THE BACTERIOLOGY OF CHRONIC ULCERATIVE COLITIS. *G. M. Dack, L. R. Dragstedt and T. E. Heinz*, Departments of Hygiene and Bacteriology, Surgery and Medicine, University of Chicago.

CURDLED BLOOD

A SIMPLY PREPARED FLUID MEDIUM FOR THE CULTIVATION OF ANAEROBES

E. MURRAY PULLAR

Walter and Eliza Hall Fellow, Veterinary Research Institute, The University of Melbourne

Received for publication February 27, 1936

Constituents. (1) Nutrient broth (pH about 7.3), 4 volumes; (2) defibrinated blood, 1 volume.

Preparation. 1. Add the defibrinated blood to the stock nutrient broth. (In this Institute the stock bouillon used contains Lemco 0.5 per cent, Witte's peptone 1.0 per cent, and sodium chloride 0.5 per cent.) The blood need not be sterile, but it is probably advisable to avoid gross contamination.

2. Mix by shaking.

3. Steam in the Arnold for 20 to 30 minutes (depending on the volume of medium being prepared). On steaming, the blood broth mixture sets to a firm gel, and changes in colour from red to a dark milk-chocolate. The flask should not be moved or bumped about during steaming as this tends to break up the clot into a muddy sludge.

4. Remove from the steamer and shake vigorously several times (prolonged shaking is unnecessary). This causes the gel to break up into small granules about 2 mm. long and 1 mm. wide.

5. Distribute into tubes and plug.

6. Autoclave at 115°C. for 20 minutes. This medium shows very little tendency to spatter or explode during sterilization. When sterilization is complete, it will be found that the granules tend to adhere, but the curd offers little or no resistance to the passage of the inoculating needle or pipette. The granules sink rapidly after agitation, and the fine débris does not stain deeply nor obscure the picture in smears stained by the ordinary methods.

The entire process should occupy one and a half to two hours.

It is unnecessary to adjust the reaction of the medium as both the ingredients are on the alkaline side of neutrality, and there appears to be very little change in reaction during preparation. Variations in the hydrogen ion concentration do not appear to influence the size of the granules of the curd. Bovine, ovine, and porcine blood has been used with equal success; but the latter appears to produce slightly larger and tougher granules. Twenty per cent defibrinated blood has been found to give the most satisfactory proportion of solid and fluid. If more or less fluid is required the percentage of defibrinated blood may be altered accordingly (10 per cent defibrinated blood gives about equal parts of curd and fluid).

This medium has been used successfully for the cultivation of a wide range of gram-positive anaerobes, including *Clostridium tetani*, *Clostridium Chauvei*, *Clostridium oedematiens*, and various types of *Clostridium botulinum* and *Clostridium Welchii*. A marked change is produced by proteolytic organisms; the curd changes in colour to dark brown or black, and is considerably reduced in volume (up to 50 per cent). Spore formation occurred with many of the strains tested, but the percentage of bacilli which sporulated was seldom large.

After a careful search of the available literature, we are led to conclude that this medium has not been previously described, although several of the preparations included in Levine and Schoenlein's (1930) compilation bear some resemblance to it. Hibler cultivated anaerobes in a medium consisting of sterile blood clot, serum and a very small quantity of bouillon or water. Griffith's medium for the cultivation of *Leptospira icterohaemorrhagiae* is prepared by mixing one part of blood with two parts of saline, and steaming it until it sets to a gel. Albert and Kelman studied toxin production by *Hemophilus influenzae* using as a medium 10 per cent defibrinated blood mixed with veal infusion broth steamed until the solution become semi-gelatinous and then allowed to settle. The medium used by Sellards and Bigelow during their attempts to cultivate measles virus was prepared

by steaming for 15 to 20 minutes a veal-liver-peptic digest to which 6 per cent blood was added.

The chief advantages of the medium here described are: (1) It can be easily and rapidly prepared from two readily available ingredients; (2) the fine debris does not interfere with the ordinary staining methods (a drawback in tissue mash anaerobic media); (3) proteolytic action is very clearly demonstrated (an advantage in class demonstration).

REFERENCE

LEVINE, M., AND SCHOENLEIN, H. W.: A Compilation of Culture Media for the Cultivation of Microorganisms. Society of American Bacteriologists. Monographs on Systematic Bacteriology, II. The Williams & Wilkins Company, Baltimore, 1930.

SEMISOLID MEDIA FOR CULTIVATION AND IDENTIFICATION OF THE SPORULATING ANAEROBES

R. S. SPRAY

From the Medical School, West Virginia University, Morgantown[1]

Received for publication February 9, 1936

This paper amplifies the studies presented serially during the past four years at the annual meetings of our Society, on the use of semisolid media as applied to the routine isolation and identification of the sporulating anaerobic bacilli. These media have been applied tentatively to a variety of aerobes as well, with indications of practical utility here also. This development, however, is a subject for later presentation.

The sporulating anaerobes are commonly cultivated in deep solid agar. Formerly such cultures were usually protected by a vaseline or other oily cap, which appears to be an unessential complication, providing the depth of medium is sufficient.

Fluid media are also routinely used for certain procedures. For these a vaseline or mechanical seal is requisite, unless incubated in an oxygen-free environment.

However, it appears that the two principles may be combined, as was probably first shown by Pringsheim (1910), by adding only sufficient agar to the fluid base to check convection currents, thus inhibiting aeration. This observation was substantiated and amplified by Jackson and Muer (1911), Lignières (1919), Hitchens (1921, 1922), Murray and Headlee (1931), among others.

When this principle is applied to a liver-peptone broth all of the common anaerobes grow abundantly in the open and un-

[1] This study has been made possible largely through the favor of a grant-in-aid of the National Research Council, to whom our appreciation is gratefully acknowledged. We are also particularly indebted to Dr. Ivan Hall, Dr. Elizabeth McCoy, Dr. Ida Bengston, Dr. Alden Rowe, and others, from whom many cultures were obtained.

protected medium. No seal or anaerobic container is required, and anaerobic cultivation under these conditions is no more complicated than that of the common aerobes. During the past five years these simple methods have been successfully applied to some 450 strains of 30 or more species of anerobes.

In general the anaerobic group is not fastidious in its nutritional requirements. However, it is observed that the butyric group is not favored by the liver-base, but grows better in a semi-solid whey-base.

Having established the practicability of this simple method of cultivation, it appears that a basic semisolid medium might be adapted to the demonstration of certain differential physiologic reactions. Thus, the production of indol, of hydrogen sulfide, reduction of nitrates to nitrites, fermentation, gelatin liquefaction, and other reactions may be determined in a medium to which the necessary ingredients and reagents are added. Suitable mediums have been devised, which are described below. The formulas given represent the optimum present development. In certain cases it is obvious that improvement is possible, and it is hoped that this report will stimulate similar studies by others.

Practical applications. We shall not discuss anaerobic isolation further than to emphasize the utility of the anaerobic culture dish described by the writer (1930). Primary cultures are inoculated into deep brain medium, or into the semisolid stock culture medium described below. After incubation these cultures are examined for spores, after which they are heated for ten minutes at 80°C., plated, and representative colonies fished to deep brain. Replating for purity is desirable.

The Hall "marble-seal" tube is most useful for anaerobic enrichment of primary cultures. Also, the addition of a tint of crystal violet to the surface of the semisolid agar, or to the supernatant fluid in the Hall tube, as advised by Hall, serves to suppress the aerobic spore-formers.

By these, and other devices, a series of pure cultures of sporulating anaerobes may be obtained. The problem of identification, however, still remains. The novice in the anaerobic field will encounter difficulty here, due chiefly to the lack of any standard

procedure of identification. Many different media and reactions have been utilized, with the result that no two descriptions of the same organisms can be made to conform. The early history and terminology are replete with descriptions of what are now recognized as mixed cultures. Multiple names for identical organisms lead to confusion, complicated further by the recent general adoption of the generic name *Clostridium* for the group.

It is one of the primary purposes, then, of this study to advocate the uniform application of a selected series of differential reactions which are of demonstrated value. These tests should be uniformly applied to all species studied. The resulting descriptions will be at least comparable, and the differentiation will be in proportion to the number and suitability of the tests employed.

Such a series of tests has been applied here to a group of 328 cultures of 20 common anaerobic species. In addition a number of other species are under study, but the present data do not warrant their inclusion in this report.

The tests, as now applied, include: (1) Motility; (2) morphology; (3) spore form and position; (4) production of hydrogen sulfide; (5) production of indol; (6, 7, 8, 9) fermentation of glucose, lactose, sucrose and salicin compared with a sugar-free control; (10) nitrate reduction to nitrites; (11) iron-gelatin liquefaction; (12) iron-milk coagulation and digestion.

It is probable that, as more species are studied, further tests may be required. Thus the butyric and Welch groups may be split into types, or sub-species, by the use of other carbohydrates, as shown by Simmonds and others.

In the "Tentative Key" below, we have attempted to arrange these tests in such order as to effect first a physiologic grouping of the unknown organism, reserving morphology, motility and sporulation as secondary—not in importance, but in availability for early recognition. Thus the reaction of *Clostridium Welchii* in iron-milk almost identifies this organism, while spore form and position may be determined at times only with the greatest difficulty. In certain species the spore position is relatively fixed, while in others it may vary from equatorial to almost terminal.

We present here the formulas as now used. The technic of

procedures is outlined, and finally the results of the study are incorporated in a "Tentative Key," which is susceptible to expansion as further species are studied.

FORMULAS

1. *Anaerobe plating (or deep-tube) medium* (or use Difco Liver-Veal Agar):

Make liver infusion; boil 50 grams Bacto Liver Powder in 1 l. of distilled water. Filter through cotton and make to 1 l. by pouring hot water through the filter. Adjust the reaction to pH 7.3 to 7.4.

To make 1 l. of agar, take

1 l. liver infusion (above)
77 grams Difco North Gelatin Agar
37 grams Difco Brain-Heart Infusion Powder
2 grams sodium nitrate

Boil to dissolve and add water to make 1 l. Retitrate, and adjust to pH 7.3 to 7.4. Autoclave in flask, and cool to solidify and to fix precipitates. When solidified, remelt and add water to make 1 l. Tube about 8 cm. deep, preferably in 200 x 15 mm. tubes. Autoclave for 15 to 20 minutes at 15 pounds pressure.

2. *Semisolid stock culture medium* (or use Difco Brain-Liver-Heart Semisolid Agar):

To make 1 l. of agar, take

1 l. liver infusion (above)
37 grams Difco Brain-Heart Infusion Powder
10 grams Bacto Tryptone
5 grams NaCl
2.5 grams agar flakes

Boil to dissolve and add water to make 1 l. Retitrate and adjust to pH 7.3 to 7.4. Filter twice through cotton with slight suction, and tube about 6 cm. deep, preferably in 150 x 13 mm. tubes. Autoclave as usual.

3. *Sugar-free fermentation base.*

To make 1 l., take

10 grams Difco Neopeptone

10 grams Difco Tryptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make 1 l. Adjust the reaction to pH 7.3 to 7.4. Andrade or other indicators may be added or omitted. These are usually reduced, although many organisms show typical fermentative reactions.

Divide into lots, and add 1 per cent of the desired sugars. Reserve one lot to be tubed without sugar as a control. Tube about 8 cm. deep in 200 x 15 mm., or better 200 x 13 mm. tubes. Autoclave as usual.

4. Nitrate semisolid agar.

To make 1 l., take
5 grams Difco Tryptone
5 grams Difco Neopeptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make to 1 l. Adjust the reaction to pH 7.3 to 7.4, then add
1 gram potassium nitrate
0.5 gram glucose

Dissolve and mix well. Tube about 8 cm. deep in 200 x 15 mm., or 200 x 13 mm. tubes. Autoclave as usual.

5. Iron-milk.

Mix fresh whole milk well and tube about 8 cm. deep in 200 x 15 mm., or in 200 x 13 mm. tubes. Add to each tube one strip of No. 26 gauge black stove-pipe iron, cut about 50 x 7 mm. Autoclave as usual, but reduce the pressure slowly when completed to avoid wetting or blowing of plugs.

6. Iron-gelatin.

To make 1 l., take
128 grams Difco Nutrient Gelatin
1 gram dextrose
1 l. distilled water

Dissolve in a double boiler to avoid scorching. Adjust the

procedures is outlined, and finally the results of the study are incorporated in a "Tentative Key," which is susceptible to expansion as further species are studied.

FORMULAS

1. Anaerobe plating (or deep-tube) medium (or use Difco Liver-Veal Agar):

Make liver infusion; boil 50 grams Bacto Liver Powder in 1 l. of distilled water. Filter through cotton and make to 1 l. by pouring hot water through the filter. Adjust the reaction to pH 7.3 to 7.4.

To make 1 l. of agar, take

1 l. liver infusion (above)
77 grams Difco North Gelatin Agar
37 grams Difco Brain-Heart Infusion Powder
2 grams sodium nitrate

Boil to dissolve and add water to make 1 l. Retitrate, and adjust to pH 7.3 to 7.4. Autoclave in flask, and cool to solidify and to fix precipitates. When solidified, remelt and add water to make 1 l. Tube about 8 cm. deep, preferably in 200 x 15 mm. tubes. Autoclave for 15 to 20 minutes at 15 pounds pressure.

2. Semisolid stock culture medium (or use Difco Brain-Liver-Heart Semisolid Agar):

To make 1 l. of agar, take

1 l. liver infusion (above)
37 grams Difco Brain-Heart Infusion Powder
10 grams Bacto Tryptone
5 grams NaCl
2.5 grams agar flakes

Boil to dissolve and add water to make 1 l. Retitrate and adjust to pH 7.3 to 7.4. Filter twice through cotton with slight suction, and tube about 6 cm. deep, preferably in 150 x 13 mm. tubes. Autoclave as usual.

3. Sugar-free fermentation base.

To make 1 l., take

10 grams Difco Neopeptone

10 grams Difco Tryptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make 1 l. Adjust the reaction to pH 7.3 to 7.4. Andrade or other indicators may be added or omitted. These are usually reduced, although many organisms show typical fermentative reactions.

Divide into lots, and add 1 per cent of the desired sugars. Reserve one lot to be tubed without sugar as a control. Tube about 8 cm. deep in 200 x 15 mm., or better 200 x 13 mm. tubes. Autoclave as usual.

4. Nitrate semisolid agar.

To make 1 l., take

5 grams Difco Tryptone
5 grams Difco Neopeptone
2.5 grams agar flakes
1 l. distilled water

Boil to dissolve and add water to make to 1 l. Adjust the reaction to pH 7.3 to 7.4, then add

1 gram potassium nitrate
0.5 gram glucose

Dissolve and mix well. Tube about 8 cm. deep in 200 x 15 mm., or 200 x 13 mm. tubes. Autoclave as usual.

5. Iron-milk.

Mix fresh whole milk well and tube about 8 cm. deep in 200 x 15 mm., or in 200 x 13 mm. tubes. Add to each tube one strip of No. 26 gauge black stove-pipe iron, cut about 50 x 7 mm. Autoclave as usual, but reduce the pressure slowly when completed to avoid wetting or blowing of plugs.

6. Iron-gelatin.

To make 1 l., take

128 grams Difco Nutrient Gelatin
1 gram dextrose
1 l. distilled water

Dissolve in a double boiler to avoid scorching. Adjust the

reaction to pH 7.3 to 7.4, and add water to make 1 l. Tube about 8 cm. deep in 200 x 15 mm., or in 200 x 13 mm. tubes. Add to each tube one strip of iron (as in Iron-Milk), and autoclave as usual.

7. Lead acetate semisolid agar.

To make 1 l., take 36 grams Difco Lead Acetate Agar (2 per cent agar). Infuse this for 30 minutes, shaking frequently, in 1 l. warm (37°C.) distilled water to dissolve all ingredients except the agar. Filter through cotton, with slight suction, to remove the agar. Make the volume to 1 l. by pouring warm water through the cotton filter. Add to this 2.5 grams agar flakes, and boil to dissolve the agar. Make the volume to 1 l., and adjust the reaction to pH 7.3 to 7.4. Tube about 8 cm. deep, and autoclave as usual.

Note: Prepared by this method the medium displays quantitatively distinctive reactions with the various anaerobes. We have been unable as yet to reproduce these distinctions by other formulas or other modes of preparation.

GENERAL PROCEDURE OF IDENTIFICATION

In this plan all intricate anaerobic apparatus, common only to research laboratories, is eliminated. Isolation, cultivation and identification are accomplished with apparatus and media readily available. Thus, anaerobic studies can be conducted by these methods in even the most meagerly equipped hospital laboratory.

The basic requirements for such studies are satisfied by:

1. The customary anaerobic jars, if available, for plate cultivation, although we favor the use of the anaerobic culture dish of the writer.[2] Deep tubes of agar may be used by those preferring this method.
2. A suitable solid plating, or deep-tube, medium (Bacto Liver-Veal Agar).
3. A semisolid stock culture medium (Bacto Brain-Liver-Heart Semisolid Agar).

[2] Obtained from Fisher Scientific Company, Pittsburgh, Pa.

4. A supply of Pasteur pipettes drawn from 3 to 4 mm. tubing.
5. Marble-seal Hall tubes for anaerobic enrichment. These are very useful, but are not required.
6. Brain medium tubes for storage of pure cultures (von Hibler's formula).
   6a. Corn-Liver medium of McClung and McCoy (1934) for storage of *Clostridium butyricum* types.
7. Special diagnostic media, chiefly semisolid, as listed above.

When assured, by replating, that pure cultures have been isolated, we establish stock cultures in deep brain, or in corn-liver. These cultures are sealed with paraffin, and are reserved in the dark at room temperature. They are opened again only as a last resort, as in case of loss or contamination of the current subcultures.

At the same time a working culture is established in the semi-solid stock culture medium (S. S. C.). Our cultures, in this medium, have all survived 54 days continuous incubation at 37°C., and all tested have been found viable after 8 months storage at room temperature. This allows ample time for all reasonable requirements. However, duplicate storage in deep brain or in corn-liver serves as a precaution against possible loss.

In preparing for the identification, a fresh subculture in S. S. C. medium is incubated not over 24 hours, in order to have active vegetative growth. A complete set of the diagnostic media is placed in a rack. If these are not used on the day of their preparation all tubes are boiled for 10 minutes and cooled just before inoculation.

The stock culture is held at a convenient angle in a burette clamp, with the mouth near a flame. A Pasteur pipette is well flamed, the sealed end snipped with flamed forceps, and the pipette is inserted and rested within the culture tube. Each diagnostic medium is then inoculated from the bottom upward with about 0.1 cc. of culture, cautiously blowing to expel the contents without bubbling air. The entire set of cultures is then incubated at 37°C.

DISCUSSION AND INTERPRETATION

1. Motility tests. It should be strongly emphasized that motility tests must be made early; in fact, preferably, as soon as growth is evidenced by perceptible turbidity. Many incorrect descriptions are based upon delay even to only 24 hours. Thus, *Clostridium bifermentans* and *Clostridium luciliae,* commonly described as non-motile, are found uniformly motile if examined at from 4 to 6 hours. Both of these species are commonly non-motile after 18 hours incubation.

No special precautions appear necessary. We remove a sample, preferably from the S. S. C. medium, by Pasteur pipette to an open slide, dropping on a cover-glass and examining as soon as convection currents subside. We have not found it necessary to seal the cover-glass with vaseline, or to examine in flattened capillary pipettes, as advocated by some workers.

The S. S. C. medium is recommended for the study of motility, although others may be used, excepting those containing fermentable carbohydrates. Negative results require, of course, repeated confirmation from a variety of media, all tests being made as early as growth is perceptible.

2. Morphology. Gram stains are commonly made at 18 to 24 hours. Because of the common morphologic similarity of the group, microscopic examination may yield little information. Even in pure cultures the limits of size and shape are such that one often cannot certify the purity. Obvious contaminants may be recognized, and certain species may be tentatively grouped. Thus, *Clostridium Welchii* may be differentiated readily from *Clostridium tertium,* or *Clostridium tetani* from *Clostridium bifermentans.*

Deep brain is, in certain respects, preferable to the S. S. C. medium for microscopic examination. In the former the morphology may be more uniform, and the Gram reaction more stable. In the latter, however, the field is entirely free from confusing debris. Unusual care is required to avoid over-decolorizing the Gram stain. Acetone alcohol must not be used as the decolorizer. The method of Claudius, as cited by Henry (1916–1917) is one of the most useful modifications.

When stained from young cultures the sporulating anaerobes appear inherently Gram-positive. Thus, this method yields little information of value, except that certain species, such as *Clostridium Welchii* and *Clostridium Sordelli*, among others, are strongly positive even in rather old cultures. This feature may then assist in the tentative grouping of unknown species.

3. Spores. We do not lay primary emphasis upon sporulation, except where it is readily observable, or where the spore has a definite and obvious shape or position. Thus, *Clostridium tertium*, with its abundant terminal spores may be readily differentiated from *Cl. Welchii* or *Cl. Sordelli*, which have central to excentric spores. *Cl. tetani* may also be distinguished from *Cl. Sordelli*, but not so readily from *Clostridium tetanomorphum*, which also has terminal spores with only a slight ovoid tendency.

Depending upon the species encountered, sporulation may or may not be particularly useful. We include it only as a secondary feature, as contrasted with its common primary position in other current systems, appreciating the fact that many will disagree. It should be noted, however, that we utilize physiology, rather than morphology, as a primary basis of classification.

4. Hydrogen sulfide. We regard this test as most useful. However, the method used at present is rather empirical. Our differentiations are based solely upon the above formula. We have been unable to obtain these quantitative differentiations when the medium is prepared by the methods used for the other semisolid mediums. The concentration of the agar appears to exert an action comparable to that recently reported by Vaughan and Levine (1936).

With our formula three distinctive reactions may be observed. Thus, *Cl. sporogenes*, *Cl. botulinum*, *Cl. Welchii* and others, show early and intense blackening at 12 to 24 hours. *Cl. tetani*, *Cl. lentoputrescens* and *Cl. tertium* show but a slight diffuse smoky browning, while *Cl. multifermentans* and *Cl. tetanomorphum*, among others, give no reaction at all.

We have experimented with the Difco Peptone Iron Agar of Levine (1932–1934), and feel that this may supplant later the lead acetate agar. However, we have not yet developed a

formula permitting the differentiation afforded by the lead acetate.

It is probable, moreover, that all of the sporulating anaerobes produce more or less hydrogen sulfide. We have developed formulas of both peptone iron and lead acetate in which all species tested blacken strongly.

5. Indol. This test is performed in the sugar-free fermentation control, after reading the pH reaction. No discrepancies have been observed between the Ehrlich and the vanillin tests. The vanillin test, however, is advocated because of a reaction described below as the "Vanillin Violet" test, which is shown by only 3 species in our series.

The minimum safe time of testing is about 72 hours. Tests may be made up to 7 days or more, with little change in reaction. Comparative tests of 12 peptones showed an obvious superiority of a combination of Difco Neopeptone and Tryptone. In this medium our strains of *Cl. tetani* and *Cl. lentoputrescens* gave a positive reaction of some degree, while negative reactions were observed in practically all other peptones tested.

When traces of glucose were added to the medium, to increase growth of the fermentative species, it appeared to exert a "protein-sparing" action on the peptones, inhibiting, or suppressing entirely, the formation of indol. In some cases it seemed that this might be due to the resulting acidity. However, *Clostridium sphenoides* gave a strong reaction in 1 per cent sucrose and lactose and a negative test in 1 per cent glucose and salicin, although all carbohydrates were fermented. Thus, it appears that the specific sugar, rather than the acidity, was the responsible factor.

"Vanillin Violet" test. When 10 drops of 5 per cent vanillin in 95 per cent alcohol are added to the culture, followed by 10 drops of concentrated HCl, the orange indol reaction appears in degree according to the species tested. Certain cultures, however, including *Cl. sporogenes*, *Cl. tyrosinogenes* and *Cl. botulinum* (*A* and *B*), showed no orange, but rather a faint violet. On further addition of the reagents this color is intensified, diffusing as a deep violet color. The nature of this reaction is not known, but

Hall suggests that it is probably due to skatol or some closely related body.

This reaction indicates, in our series, one of these three species, and it is interesting to note that it occurs in mixed as well as in pure cultures. Thus, it was observed in a culture of *Cl. Welchii* containing *Cl. sporogenes* as a contaminant. This test may, then, be of value in checking cultures for freedom from *Cl. sporogenes*, probably the most common anaerobic contaminant.

6, 7, 8, 9. Fermentation tests. We have used, thus far, only four carbohydrates. Others may be required later as other species are studied. For example, the *Cl. butyricum* group seems to include a variety of fermentative types, whose reactions may later prove correlated with other cultural distinctions.

We utilize only the end reaction as evidence of fermentation. Presence and amount of gas cannot be relied upon, as emphasized by Hall, Heller and others. We do not use vaseline caps, or attempt any measurement of gas. Of course, great excess of gas and growth, as contrasted with the sugar-free control, is indicative. It may often serve as a fairly reliable index, but nothing more.

In general, 36 hours incubation appears to be the optimum time for testing acidity. However, certain species, which grow rather poorly, continue slowly to increase in acidity up to at least 7 days. This may explain some of the discrepancies in past studies of *Clostridium Chauvoei*, *Clostridium fallax* and other rather feebly growing anaerobes.

We have tried adding various indicators to the medium. However, as observed by others, these are commonly reduced to an inactive form. By reducing the peptone content some indicators remain reasonably stable and active, but as the peptones are reduced in amount many species, such as *Cl. Chauvoei* and *Cl. Novyi*, may fail to grow. Hence, we have discontinued their use in the medium, and follow Hall's procedure using the spot plate, or remove aseptically by pipette some 5 drops of culture and test this in tubes of properly diluted indicator. If carefully done, the culture may be reincubated for subsequent retest.

There is no purpose in reading the exact pH in routine iden-

tification. A definite acid reaction, as contrasted with the sugar-free control, is sufficient indication of fermentation. With few exceptions the reaction is sharp and obvious. In such exceptions a repetition of the test at 24 to 48 hours later usually confirms the reaction.

10. Nitrate reduction to nitrites. This test, usually considered a simple one, has given considerable difficulty. This has been largely overcome by the present formula of the medium. The difficulty is apparently due to several factors which require a delicate balance, as recently summarized by Conn (1936).

Thus, in order to obtain the growth requisite for reducing the nitrate, it is almost necessary to add a trace or more of glucose. Yet, when this is added to facilitate growth of the fermentative types, nitrate reduction may be distinctly, or completely, suppressed.

To compensate for this inhibition we have added larger amounts of peptone. Here, again, a second inhibition is observed. An excess of peptone, as well as traces of glucose, seriously interferes with the accuracy of the test. Moreover, of several test organisms, each responds differently to the varying concentrations of each factor. For example, *Cl. Welchii*, *Cl. multifermentans*, *Cl. tertium* and *Cl. septicum* all behave differently from each other.

In the formula above, however, the peptone, glucose and nitrate have been so adjusted that uniformly positive reactions are obtained. We believe that this formula may yet be improved, and are working toward this end.

The test is usually performed after 72 hours incubation, although the reaction does not vary thereafter up to 7 days. We prefer the use of 0.6 per cent dimethyl-alpha-naphthylamine (Eastman), with sulfanilic acid, both in dilute acetic acid.

11. Iron-gelatin liquefaction. This test is performed in Difco Nutrient Gelatin to which is added 0.1 per cent of glucose and a strip of iron.[3] The Standard Methods gelatin formula is to be avoided. In this simple gelatin solution many anaerobes fail to

[3] Powdered iron reduced by hydrogen may be used, as advocated for milk by Hastings and McCoy (1932), although we prefer the iron strip, as suggested by H. G. Dunham of Difco Laboratories.

liquefy in any reasonable time, while in our formula the same organisms commonly liquefy completely in 24 to 48 hours. This is particularly striking in the case of *Cl. septicum* and *Cl. luciliae*.

All cultures are incubated at 37°C., and are tested daily in ice water until liquefied, or, if negative, at least for 30 days. No time limit can be set for the negative cultures, but we have not observed any species showing liquefaction delayed beyond 10 days. If incubated beyond 10 days, the plugs should be paraffined.

The iron-gelatin shows a distinctive orange to wine-red color when inoculated with *Clostridium histolyticum*. The orange color appears at about 24 hours, deepens to red at about 48 hours, then slowly and variably fades in 3 to 5 days or more. The reaction appears specific for *Cl. histolyticum*, and establishes a valuable presumptive test for this organism, having the same significance as the reaction of *Cl. Welchii* in iron-milk. The reaction is displayed in mixed, as well as in pure cultures, but its particular value lies in its application to pure cultures.

12. Iron-milk. The reactions in this medium are utilized for the primary group differentiation. As noted above, Hastings and McCoy used powdered reduced iron for the detection of *Cl. sporogenes* in milk. For a time we used this with satisfaction, until the idea of the iron strip was suggested by Dunham. The results are so distinctly superior that this principle has been adapted to our purpose.

This medium affords 5 distinctive and useful reactions, which permit ready grouping of the unknown strains. With some species the reaction is striking and early; in others, especially the non-reactors, delay is necessarily encountered. However, failure to react has the usual value of other negative tests.

These reactions have been so often repeated that one may conclude that they are uniformly characteristic, and practically unvarying in reliability. Even such selective organisms as *Cl. Chauvoei*, *Cl. luciliae* and *Cl. Novyi* present no difficulty of cultivation in this simple medium. No vaseline seal, or other anaerobic conditions are required for the cultivation of any of our species.

The group reactions in iron-milk and their interpretation

I. Active gaseous fermentation, with early coagulation (12 to 48 hours). The clot is violently disrupted, with no subsequent digestion or blackening. This reaction suggests commonly *Cl. Welchii*, although other anaerobes, including *Cl. aerofoetidum*, *Cl. multifermentans* and members of the *Cl. butyricum* group, approach or even equal it in intensity.

As was repeatedly observed by Hall, this "stormy" reaction in milk inoculated with material from gangrenous wounds, or other positive sources, suggests the presence of *Cl. Welchii*. However, it is indicative only, in iron-milk, since several other anaerobes may cause a similar reaction.

II. Inactive gaseous fermentation, with coagulation quite uniformly delayed (4 to 6 days). No digestion or blackening of the clot is observed, although a dirty gray-brown discoloration may occur if evaporation exposes the end of the iron strip. Such changes are of no significance. Furthermore, cultures requiring prolonged incubation should have the plugs paraffined after the first 5 to 6 days.

In this group we find *Cl. sphenoides*, *Cl. fallax*, *Cl. tertium* and *Cl. septicum* showing a fine stream of small gas bubbles rising in 12 to 24 hours. This gas evolution continues for several days, never becoming more than barely perceptible. Upon shaking the tube gently a "shower" of bubbles is released to rise in a fine foam.

Coagulation occurs suddenly and almost uniformly in 5 to 6 days, but, since most of the gas has already escaped, the clot shows only a slight splitting. It is never torn as in the preceding group. If the plugs are paraffined no further change, other than a slight graying, is observed for many days or weeks.

III. Inactive gaseous fermentation, long continued, with late (if any) coagulation; no digestion or blackening. This group differs from group II only in that coagulation is uncommon, and rarely appears before 12 days, if at all. Fermentation is very slight, being evidenced often by only single bubbles which arise only on shaking. The evolution of gas, however, is indefinitely prolonged, gas bubbles being observed after as long as 60 days incubation.

In this group we place *Cl. Novyi*, *Cl. Chauvoei* and *Cl. luciliae*.

IV. Inactive gaseous fermentation, with digestion and blackening. This reaction is almost as striking as the "stormy" reaction of *Cl. Welchii*. In 12 to 24 hours the milk becomes watery and more or less translucent. A soft semi-coagulation may then occur (*Cl. botulinum A* and *B*), or it does not (*Cl. sporogenes*, *Cl. histolyticum* and *Cl. bifermentans*). Little or no gas is observed, at most only an occasional bubble arising on shaking.

After the initial translucence, and up to 5 to 7 days, according to the species, a fine, then coarse flocculence, or a soft coagulation develops. This darkens with variable rapidity, often becoming coal-black within 24 hours (*Cl. sporogenes*). Digestion proceeds, and fine or coarse flocculi settle as a black sediment, leaving a yellow to greenish supernatant fluid. Paraffined tubes ultimately become almost clear, with only a small amount of black precipitate.

In general, these changes are rapid and complete within 5 to 6 days, except for the prolonged settling. This excepts *Cl. botulinum A* and *B*, which usually clot the milk softly in 2 to 5 days, with blackening delayed until 5 to 7 days, after which further changes follow those of the other members of the group. This coagulation and delayed digestion, at least among our 13 strains, seems to be a reliable index of the species. Together with fermentation of salicin and the "Vanillin Violet" reaction, it suffices to identify the species.

V. No gaseous fermentation, with no digestion or blackening. Coagulation is rather constant, but long delayed, by *Cl. tetani* and *Cl. lentoputrescens*. This is not an acid coagulation, but is due rather to a weak coagulase. It appears, at the earliest, in from 15 to 20 days. A few strains have coagulated at 30 days or later, while others have failed to coagulate even after some 5 months incubation.

The fact that these cultures are growing actively is easily demonstrable by microscopic examination, or by subculture. The negative early milk reactions are readily supported by the other reactions, such as indol formation, gelatin liquefaction and failure to ferment carbohydrates.

Cl. tetanomorphum follows the same negative course in iron-

milk. However, it ferments glucose and gives a very striking indol reaction. Our strains have shown no tendency to coagulate iron-milk, even after 60 days incubation. This adds a belated confirmation of its identity. Its ovoid spores offer further differentiation from *Cl. tetani*.

The results of all these reactions and observations are presented in the form of a "Tentative Key."

"TENTATIVE KEY" TO THE SPORULATING ANAEROBES

I. Iron-Milk, Active gaseous fermentation; Early coagulation (12–48 hours); No digestion of clot; No blackening;
  A. Lead Acetate, Strongly blackened;
    1. Sucrose, Fermented; (Salicin,—Not fermented);
      Nitrite +; Indol −; Violet −; Gelatin +; Motility −;
        Glucose +; Lactose +;
          Spores ovoid, central-excentric; Not swelling rod;
            (Spores infrequently observed, − not in fermentable sugars);
              .....1. *Clostridium Welchii*
                (44 cultures)

    2. Sucrose, Not fermented; (Salicin, − Fermented);
      Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
        Glucose +; Lactose +;
          Spores ovoid, excentric-subterminal; Distinctly swelling rod;
            (Spores infrequently observed, apparently not in fermentable sugars);
              .....2. *Clostridium aerofoetidum*
                (1 culture)

  B. Lead Acetate, Not blackened; No browning;
      Nitrite +; Indol −; Violet −; Gelatin −; Motility +;
        Glucose +; Lactose +; Sucrose +; Salicin +;
          Spores ovoid, central-excentric; Not swelling rod;
            (Spores abundant, even in fermentable sugars);
            a. Glycerol, − Fermented;
              .....3. *Clostridium multifermentans*
                (13 cultures)

            b. Glycerol, − Not fermented[4]
              .....4. *Clostridium butyricum* (Group)
                (11 cultures of various species)

II. Iron-Milk, Inactive gaseous fermentation; Late coagulation at 4–6 days; No digestion of clot; No blackening;
  A. Lead Acetate, − Strongly blackened;

---

[4] Our strains are in accord with the common impression that members of the group do not ferment glycerol. We are advised, however, by McCoy, that this is not entirely and uniformly true.

1. Indol, Positive;
Nitrite +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +;[4] Salicin +;
([4] pH in Sucrose reduced from 7.2 to 6.4 only);
Spores almost spherical, abundant, subterminal, becoming terminal; Swelling rod;
Vegetative cells distinctly navicular, pointed;
.....5. *Clostridium sphenoides*
(1 culture)

2. Indol, Negative;
Nitrite +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +; Salicin +;
Spores ovoid, excentric-subterminal; Swelling rod;
(Spores infrequently observed, not abundant);
Vegetative cells slender, round to pointed ends;
.....6. *Clostridium fallax*
(1 culture)

B. Lead Acetate, Slight smoky browning; Not blackened;
Nitrite +; Indol −; Violet −; Gelatin −; Motility +;
Glucose +; Lactose +; Sucrose +; Salicin +;
Spores ovoid, terminal; Swelling rod;
(Spores abundant, even in fermentable sugars);
Grows aerobically, (Microaerophile);
.....7. *Clostridium tertium*
(9 cultures)

C. Lead Acetate, No blackening; No browning;
Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Lactose +; Sucrose −; Salicin +;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
.....8. *Clostridium septicum*
(Vibrion septique)
(*Clostridium oedematis-maligni*)
(92 cultures)

III. Iron-Milk, Inactive gaseous fermentation (long continued);
Late, if any, coagulation (10–20–30 days, or not even at 60 days); No digestion; No blackening;
A. Lead Acetate, Strongly blackened;
1. Lactose, Not fermented;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Sucrose −; Salicin −;
Spores ovoid, not abundant, excentric-subterminal;
Swelling rod;
(Some few strains coagulate Iron-Milk (5–12 days), others not 30 days, although gas bubbles are constantly evolved);
.....9. *Clostridium Novyi*
(*Clostridium oedematiens*)
(28 cultures)

2.
+ Lactose, Fermented;
Nitrite +; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Sucrose +; Salicin −;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
(Some strains coagulate Iron-Milk (12 days); others not at 56 days);
.....10. *Clostridium Chauvoei*
(7 cultures)

B. Lead Acetate, No blackening; No browning;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −;
Spores ovoid, not abundant, terminal; Slightly swelling rod;
(No coagulation of Iron-Milk (30 days), although gas bubbles are constantly evolved (15–20 days));
.....11. *Clostridium luciliae*
(*Clostridium botulinum* C)
(9 cultures)

IV. Iron-Milk, Inactive gaseous fermentation; More or less rapid digestion (with or without previous clotting); Strongly blackened, early (48 hours) or late (8–9 days);
A. Lead Acetate, Strongly and rapidly blackened (24–48 hours);
1. Salicin, − Fermented;
Nitrite −; Indol −; Violet +; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −;
Spores ovoid, not usually abundant, excentric-subterminal; Swelling rod;
(Iron-Milk softly coagulated (2–5 days); First blackened (5–7 days); Clot slowly digested (10–20 days));
(Differentiated by toxin-antitoxin);
.....12. *Clostridium botulinum* A and B
(13 cultures)

2. Salicin, Not fermented; (See *Clostridium centrosporogenes*);
a. Indol, − Negative; (Vanillin Violet +);
Nitrite −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −;
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;
(Iron-Milk not coagulated; Translucent, then flocculent precipitate; Rapidly blackened (24–48 hours); Rapidly digested (8–10 days));
1. Tyrosine crystals not observed;
.....13. *Clostridium sporogenes*
(11 cultures)

2. Tyrosine crystals in old cultures;
.....14. *Clostridium tyrosinogenes*
(4 cultures)

b. Indol, Positive; (Vanillin Violet −);
Nitrite −; Gelatin +; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −;[5]
Spores ovoid, abundant, central-excentric;
Not markedly, if at all, swelling rod;
1. Pathogenic;

.....15. *Clostridium Sordelli*
(10 cultures)

2. Non-pathogenic;

.....16. *Clostridium bifermentans*
(*Clostridium centrosporogenes*)[5]
(23 cultures)

B. Lead Acetate, Not blackened; No browning;
Nitrite −; Indol −; Violet −; Gelatin +; Motility +;
(Wine-red color in Iron-Gelatin (24–48 hours))
Glucose −; Lactose −; Sucrose −; Salicin −;
(No sugars fermented)
Grows aerobically, (Microaerophile);
Spores ovoid, abundant, excentric-subterminal;
Swelling rod;

.....17. *Clostridium histolyticum*
(21 cultures)

V. Iron-Milk, No gaseous fermentation; No digestion; No blackening; Coagulation late, if any, (15–20 days or more);
A. Lead Acetate, Not blackened, but showing smoky browning at 24–48 hours, not measurably increased on incubation;
Nitrite −; Indol +;[6] Violet −; Gelatin +; Motility +;
Glucose −; Lactose −; Sucrose −; Salicin −; (No sugars fermented);
Spores spherical, not abundant, terminal;
Swelling rod;
a. Toxic;

.....18. *Clostridium tetani*
(15 cultures)

b. Non-toxic;

.....19. *Clostridium lentoputrescens*
(*Clostridium putrificum*)
(10 cultures)

B. Lead Acetate, Not blackened; No trace of browning;
Nitrite −; Indol +; Violet −; Gelatin −; Motility +;
Glucose +; Lactose −; Sucrose −; Salicin −[7]
Spores spherical to perceptibly ovoid, terminal;
Swelling rod;

.....20. *Clostridium tetanomorphum*
(5 cultures)

---

[5] (5 of 13 cultures, labeled *Cl. centrosporogenes*, frankly fermented *salicin*.)
[6] Occasional strains are Indol-weak, or apparently Indol-negative.
[7] Some strains lower pH slightly (from 7.2 to 6.8).

SUMMARY

This report, based upon some 5 years study of 328 strains of 20 species of the sporulating anaerobes, proposes the use of a series of selected physiologic tests, supported by morphologic characters, which tests should be applied uniformly to all unknown species of the anaerobe group.

These tests are applied chiefly in semisolid media which permit abundant growth under conditions eliminating all of the tedious routine of the customary anaerobic technic.

Formulas for these media, methods of performing tests, and interpretations of the reactions are outlined.

Certain newly observed reactions are recorded, which are valuable in the tentative recognition of a few species.

These reactions and observations are arranged in a definite plan, and a "Tentative Key" to 20 common species is presented. In most instances these suffice to identify the species. In a few cases they offer only "group" identification, as of the butyric or Welch group.

It is obvious that this "Tenative Key" is open to modification and addition of other reactions as more species are studied.

Some five years of study have amply demonstrated the utility of these methods. Their simplicity makes possible the easy and certain cultivation and identification of the sporulating anaerobes even in the small laboratory, and by relatively inexperienced technicians. Such, at least, is the opinion offered by other schools and laboratories which have adopted these methods, even in their present state of development.

It should be emphasized that this report is presented, not as a completed study, but rather as one indicating a new form of approach to the problem of cultivating a group of bacteria commonly regarded as especially difficult to deal with. Many species remain to be studied, particularly the thermophiles, as well as the confusing butyric group.

REFERENCES

Conn. 1936 Jour. Bact., **31**, 325.
Hastings and McCoy. 1932 Jour. Bact., **23**, 54.

HENRY. 1916–1917 Jour. Path. and Bact., **21**, 380.
HITCHENS. 1921 Jour. Infect. Dis., **29**, 390.
HITCHENS 1922 Abstr. Bact., **6**, 36.
JACKSON AND MUER. 1911 Amer. Jour. Pub. Health, **1**, 927.
LEVINE. 1932 Proc. Soc. Exper. Biol. and Med., **29**, 1022.
LEVINE. 1934 Amer. Jour. Pub. Health, **24**, 505.
LIGNIÈRES. 1919 Compt. Rend. Soc. Biol., Paris, **82**, 1091.
MCCLUNG AND MCCOY. 1934 Jour. Bact., **28**, 267.
MURRAY AND HEADLEE. 1931 Jour. Infect. Dis., **48**, 438.
PRINGSHEIM. 1910 Ctrlbl. f. Bakt., II Abt., **26**, 222.
SPRAY. 1930 Jour. Lab. and Clin. Med., **16**, 203.
VAUGHAN AND LEVINE. 1936 Jour. Bact., **31**, 24.

ESSENTIAL GROWTH FACTORS FOR PROPIONIC ACID BACTERIA[1]

I. SOURCES AND FRACTIONATION

E. L. TATUM, W. H. PETERSON AND E. B. FRED

Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin

Received for publication March 26, 1936

In a previous paper Fromageot and Tatum (1933) described a heat-stable activator essential for the vigorous fermentation of glucose and the production of acid by certain propionic acid bacteria in a corn-mash glucose medium. This medium was selected because of its low content in soluble nitrogen and because it was almost entirely lacking in the stimulating factor, which was found in the juice of the potato. It should be emphasized that the factor from potato was more effective in activating the fermentation of glucose by a non-starch-fermenting strain of propionic acid bacteria than it was in activating the fermentation of starch by a starch-fermenting strain or the fermentation of calcium lactate by either strain. The most important function of the stimulant seemed to be the activation of the fermentation of glucose. It is also of note that there was, in all cases, a perfect correlation between glucose fermented and acid produced.

This paper deals with the general physiological requirements of the propionic acid bacteria and with the fractionation of the stimulant from potato.

EXPERIMENTAL

Preliminary investigations

Culture and medium. Throughout this work *Propionibacterium pentosaceum* no. 11 was used. This organism is a non-starch-

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

fermenting strain which had been used in the previous investigation (Fromageot and Tatum (1933)). The medium was, in most cases, a 1 per cent corn mash to which was added 1 per cent glucose. The growth on this medium was very scant. The fermentations were carried out in test tubes, 1 inch in diameter, containing 35 cc. of medium. Each tube was inoculated with 0.3 cc. of a 48-hour culture of the organism grown in a medium containing 1 per cent malt-sprouts, 1 per cent glucose and calcium carbonate. The stimulation brought about by the various preparations was estimated by titration of the acid produced after seven to ten days' incubation at 30°C. Brom-thymol-blue was used as an indicator. It was not thought necessary to determine the amount of unfermented sugar, since previous work had shown a complete accord between sugar destroyed and acid produced.

Preparation of potato extract. The crude material was prepared by pressing ground white potatoes. The protein was removed from the juice by heating to coagulation and filtering. Six bushels of potatoes (165 kgm.) gave 45 liters of juice which were concentrated on the steam bath to 16.5 liters. The stock solution and the various fractions prepared from it were kept corked under toluene to prevent spoilage. The same stock solution of crude stimulant was used for all investigations.

Effect of crude extract on fermentation. The effect of increasing amounts of the crude preparation is shown in figure 1. The potato series were analyzed after 7, 8 and 10 days; the others after 10 days. The optimum concentration of extract, about 3 cc. per 100 cc. of medium, produced after 7 days' fermentation about three times as much acid as the control while after 10 days the acid rose to four times that in the control. In order to shorten the time required to obtain the results, most of the succeeding fermentations were analyzed after 6 or 7 days.

Sources of the stimulant other than potato. According to Wilson, *et al.* (1930) the propionic acid bacteria grow well on yeast water or molasses media. These substances were tested for their ability to replace potato extract in the basal corn-mash medium and to find out if they might serve as better sources of the stimulant

than potato for isolation purposes. Orange juice was also tested. The results are shown in figure 1. Orange juice was

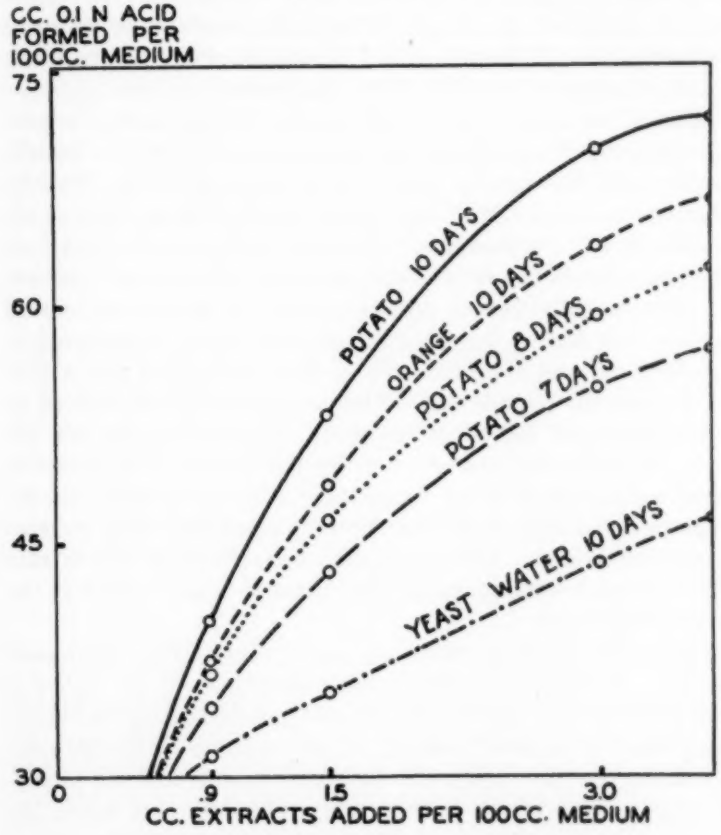


FIG. 1. EFFECT OF VARIOUS PREPARATIONS ON PRODUCTION OF ACID BY CULTURE 11

Orange, 1 cc., is equivalent to 10 cc. fresh juice; 1 cc. yeast water is equivalent to 10 grams fresh yeast; 1 cc. potato is equivalent to 10 grams fresh potato. (Note: 15.2 cc. 0.1 N acid was produced in the basal medium alone.)

found to contain the stimulant, but in lower concentrations than potato. Yeast water was also less active than potato, probably

because of a lower stimulant content. Molasses had no stimulating action whatever and seemed to be toxic for the organism in the medium and concentrations used. These negative results are not shown on the graph. Since potato extract is probably the best source of the factor and is most readily and easily obtained, it has been used throughout the succeeding investigation.

Effect of asparagine on the propionics. Potato extract stimulates not only *Propionibacterium pentosaceum* No. 11 but certain butyric acid bacteria as well. In a previous study (Tatum, Peterson and Fred (1935)) the active principle in potato extract was shown to be asparagine.[2] Aspartic and glutamic acids had the same stimulating action as asparagine. To discover whether the stimulating action of potato extract on the propionic acid bacteria was due to the same factors, the effect of asparagine, aspartic acid, and glutamic acid on these organisms was tested. These three compounds were added in concentrations of from 15 to 120 mgm. per 100 cc. of medium. Sixty milligrams per 100 cc. is the optimum concentration for the butyric acid bacteria. These substances had no stimulatory action whatever on the propionics, the acid production actually decreasing with increasing concentrations. This shows that the factor in potato extract which stimulates the propionic acid bacteria is not related to the butyric acid factor.

Stimulating effect of potato in malt-sprouts calcium-carbonate medium. There are several possible objections to the use of a basal medium such as corn-glucose and to determining the stimulating effect of an added complex organic mixture by the titration of acid produced. When the medium contains so little nitrogenous material available to the organism as does corn mash, the increase in acid production caused by the addition of nitrogenous material such as potato extract might be due to the nitrogen added rather than to a specific stimulating factor. The stimulation might also be due to an increased buffering effect of the added extract.

[2] Asparagine stimulates only certain strains of butyric acid bacteria. The strains which respond belong in the isopropyl alcohol-producing type in the grouping of these organisms proposed by Langlykke, *et al.* (1935). All strains which showed little or no response belong to the acetone-producing type.

In order to determine whether the stimulation by potato extract was due primarily to buffering or to the added nitrogen, a 3 per cent malt-sprouts medium which contains relatively large quantities of soluble nitrogen was used as a basal medium. Three per cent glucose and an excess of calcium carbonate were added. After fermentation the acid produced was determined by steam-distilling an aliquot acidified with sulphuric acid. The effect of the addition of potato extract to this medium is shown in table 1. The effect was most noticeable after 5 days. With the same concentration of potato that was found to be optimum on corn-mash (*i.e.*, 3 cc. per 100 cc. of medium) the production of acid was four times that in the control. After ten days the

TABLE 1

Effect of potato extract on acid production in a glucose malt sprouts medium

POTATO EXTRACT ADDED PER 100 CC. MEDIUM	LENGTH OF FERMENTATION	ACID PRODUCED PER 100 CC. MEDIUM
cc.	days	cc. 0.1 N
0.0	5	33.0
0.9	5	67.0
1.5	5	94.0
3.0	5	139.0
0.0	10	130.0
0.9	10	198.0
1.5	10	210.0
3.0	10	258.0

control had increased so that the addition of potato only doubled the acid production. The decided stimulatory effect on this nitrogen-rich, well-buffered medium indicates that potato extract contains a true stimulatory factor and that its activity is not due primarily to its available nitrogen or its buffering capacity, although as will be shown in a later paper (Tatum, Wood and Peterson (1936)), the character of the nitrogen supplied does have a definite significance. The amount of acid developed in the malt-sprouts control as compared with the corn-glucose control is probably due partly to the stimulant content of the malt-sprouts and partly to the neutralizing effect of the calcium carbonate.

Function of corn in the medium. When the concentration of

potato in the corn-glucose medium was kept constant and the amount of corn varied, it was found that the corn supplied something essential for growth and fermentation. Accordingly, water and alcohol extracts of corn were prepared. The water extract was prepared by soaking ground corn in hot water for about an hour and filtering. The alcohol extract was prepared by heating a suspension of ground corn in 95 per cent alcohol on the steam bath for 12 hours. The filtrate was concentrated to a syrup, poured into water and the precipitated zein filtered off. These two extracts were tested for the corn factor by addition to a medium containing 1 per cent glucose and 3 cc. of potato extract per 100 cc. The results of these experiments are shown in table 2.

TABLE 2

Effect of corn and corn extracts on acid production in glucose potato extract medium

CORN OR CORN EXTRACT ADDED TO 100 CC. MEDIUM	LENGTH OF FERMENTATION	ACID PRODUCED PER 100 CC. MEDIUM
	days	*cc. 0.1 N*
None	7	5.0
1 gram corn	7	60.0
Water extract of 1 gram corn	7	59.0
Alcohol extract of 1 gram corn	7	24.0
Alcohol extract of 3 grams corn	7	36.0
Alcohol extract of 5 grams corn	7	50.0

Both extracts were effective in replacing the crude corn. The water extract was best, since it had a maximum effect at a concentration corresponding to one per cent of whole corn. The alcohol extract was about one-fifth as effective, since a concentration representing 5 per cent of corn was required to bring about the same fermentation as did 1 per cent crude corn or water extract of corn. The investigation of the corn factor is being continued with the aim of isolating and identifying the essential constituent.

Nature of the factor in potato

Stability of the factor in potato. The factor in potato was shown to be stable to acid and alkali under the following conditions. Two samples of crude extract were autoclaved for one hour at 20

pounds pressure with equal volumes of normal sulphuric acid and normal sodium hydroxide respectively. The treated samples

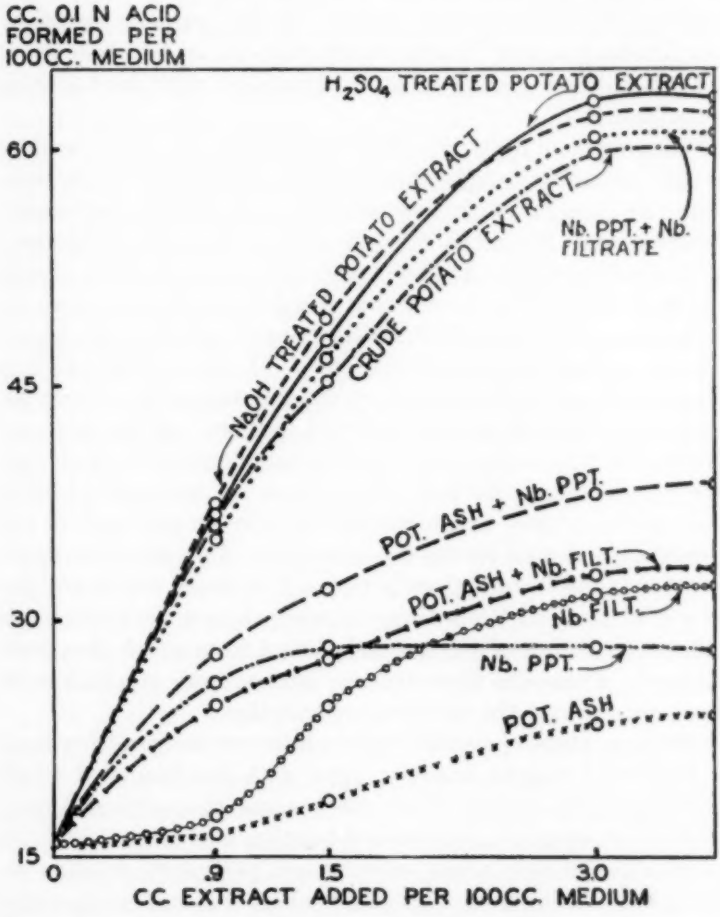


FIG. 2. EFFECT OF FRACTIONATIONS AND TREATMENTS OF POTATO EXTRACT ON ACID PRODUCTION BY CULTURE 11
(Length of fermentation, 7 days)

were then neutralized and tested for stimulation. The results are shown in figure 2. Neither treatment had much effect on the

stimulatory property; thus, a great many substances, including most oxidation reduction poising agents, are eliminated as possible causes of the stimulation.

Fractionation of the active extract. In the previous investigation (Fromageot and Tatum (1933)) the extract had been freed of carbohydrates and proteins by treatment with lead acetate and ammonia.

This treatment did not affect its action. It was therefore thought advisable to try a more efficient amino-acid precipitant. Neuberg's reagent (mercuric acetate and sodium carbonate) (Neuberg and Kerb (1912)) was chosen since it precipitates amino-acids, peptides, peptones and similar compounds almost quantitatively. This reagent had been used in a previous investigation of the potato extract for the precipitation of a stimulant for certain butyric acid bacteria (Tatum, *et al.* (1935)). Through the use of this reagent on crude potato extract two fractions were obtained, the precipitated portion, and the non-precipitated or filtrate fraction. Both fractions were freed of mercury by treatment with H_2S, filtered from the mercuric sulphide, made up to volume, and tested for activity on the basis of the original extract used for the fractionation. As is shown in figure 2 neither of the two fractions is very active alone, but when they are recombined, all the activity is restored, and the stimulation is the same as that of the original extract from which they were obtained. Evidently there are two factors, both of which must be present to cause the maximum stimulation.

This separation of potato extract into two essential fractions by Neuberg's reagent does not agree with the results of Chaix and Fromageot (1935). With this reagent they obtained from potato a nitrogenous, precipitated fraction which was active in the presence of potato ash, whereas our precipitate-fraction required an additional organic factor which was not precipitated and which could not be replaced by potato ash, as will be shown later. This discrepancy can not be adequately explained. It is possible that the potato extract which they obtained contained both of our organic factors in such a combination or state that both were completely precipitated by Neuberg's reagent.

The effect of ash of potato extract. Since the Neuberg filtrate fraction contains the mineral salts of the extract they might be responsible for its activity. In order to test this possibility an ash solution was prepared by igniting a definite volume of potato extract, treating the ash with an excess of acetic acid, neutralizing the excess acid and making the solution up to the volume of the original extract taken. The effect of this potato extract ash alone and with the two active Neuberg fractions was investigated. The results of the fermentations are shown in figure 2. The addition of ash to the Neuberg filtrate had no significant effect, while it did have a slight effect when used by itself and when added to the Neuberg precipitate. This indicates that the ash elements are of some significance, and may account for part of the activity of the filtrate fraction. However, this experiment also proves conclusively that the Neuberg filtrate fraction contains some accessory substance other than the ash, since the ash can not replace this fraction. An investigation of the effect of the various ash elements is being carried out.

DISCUSSION

The stability of crude potato extract to acid and alkali treatment and the fact that none of the activity is lost on treatment with lead acetate and ammonia, shows that both essential factors in the potato extract are relatively simple and stable substances. Furthermore, neither of the potato factors can be of carbohydrate nature since all carbohydrates are precipitated by lead acetate and ammonia. The Neuberg precipitate fraction undoubtedly owes its activity to some nitrogenous substance or substances, probably amino acids or related compounds. On the other hand, the Neuberg filtrate fraction is probably not either carbohydrate or amino acid in character. Compounds of the latter type are generally assumed to be precipitated by Neuberg's reagent. The mineral salts of the potato extract which are contained in the Neuberg filtrate fraction, seem to have some significance, but can not be thoroughly studied until the essential stimulant in the Neuberg filtrate can be freed of inorganic salts.

Further work is being carried out on the purification and sepa-

ration of the above fractions in the hope that it will be possible to identify the essential substances and to determine the rôle that each plays in the fermentation of glucose by propionic acid bacteria.

One of the potato fractions, the Neuberg precipitate, is the subject of the following paper (Tatum, Wood and Peterson, (1936)).

SUMMARY

Potato extract, orange juice and yeast water have been found to stimulate the fermentation of glucose and the production of acid by *Propionibacterium pentosaceum* No. 11. The potato extract was active only in the presence of another factor which could be supplied by corn or by water or alcohol extracts of corn.

The stimulating action of potato extract is probably due to true essential growth factors and not primarily to the available nitrogen or buffering capacity of the extract.

The potato extract can be separated into two fractions, both of which must be present to bring about the stimulating effect. Neither fraction loses its activity on mild treatment with sulphuric acid or sodium hydroxide, and neither is of carbohydrate nature. One fraction is precipitated by Neuberg's reagent and may be composed of amino acids or similar compounds. The other is contained in the filtrate from the Neuberg precipitate and is apparently not amino acid in character.

REFERENCES

CHAIX, P., AND FROMAGEOT, CL. 1935 Bul. Soc. Chim. Biol., **17**, 874.
FROMAGEOT, CL., AND TATUM, E. L. 1933 Biochem. Ztschr., **267**, 360.
LANGLYKKE, A. F., PETERSON, W. H., AND MCCOY, E. 1935 Jour. Bact., **29**, 333.
NEUBERG, C., AND KERB, J. 1912 Biochem. Ztschr., **40**, 498.
TATUM, E. L., PETERSON, W. H., AND FRED, E. B. 1935 Jour. Bact., **29**, 563.
TATUM, E. L., WOOD, H. G., AND PETERSON, W. H. 1936 Jour. Bact., **32**, 167.
WILSON, P. W., FRED, E. B., AND PETERSON, W. H. 1930 Biochem. Ztschr., **229**, 271.

ESSENTIAL GROWTH FACTORS FOR PROPIONIC ACID BACTERIA[1]

II. NATURE OF THE NEUBERG PRECIPITATE FRACTION OF POTATO: REPLACEMENT BY AMMONIUM SULPHATE OR BY CERTAIN AMINO ACIDS

E. L. TATUM, H. G. WOOD[2] AND W. H. PETERSON

Departments of Agricultural Chemistry and Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin

Received for publication March 26, 1936

The preceding paper (Tatum, Peterson, and Fred (1936)) demonstrated that the potato stimulant for the propionic acid bacteria could be separated into two fractions by Neuberg's reagent. These fractions were inactive unless combined. It was also shown that on the medium studied at least three distinct substances were required by the organisms for the vigorous fermentation of glucose. These included factors supplied by (1) corn extract, (2) the Neuberg filtrate of potato extract, and (3) the Neuberg precipitate of potato extract. The present paper deals with the nature of the last mentioned fraction, the nitrogenous Neuberg precipitate.

EXPERIMENTAL

The same technique was used as in the previous work. A corn-mash glucose or a corn-extract glucose medium was employed with culture *Propionibacterium pentosaceum* no. 11. The Neuberg filtrate was added to these media in the optimum concentration (3 cc., representing 30 grams of raw potato, per 100 cc. medium). The effect of various substances used in place of the Neuberg precipitate was then noted. Before sterilization the medium was adjusted to a pH of 7.0. The stimulating effect of

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

[2] National Research Fellow in the Biological Sciences.

the substances tested was in most cases measured by determining the acid produced.

Hydrolysis of the Neuberg precipitate. In order to determine whether the active constituent of the Neuberg precipitate was possibly an amino acid or a more unstable nitrogenous substance, the stability of this fraction to hydrolysis with strong sulphuric acid was tested. The sample was hydrolyzed in the autoclave for fifteen hours with 30 per cent sulphuric acid by weight. The sulphuric acid was removed with barium hydroxide, the liquid was concentrated to its original volume and tested.

TABLE 1

*Effect of hydrolysis of Neuberg precipitate and its replacement by fractions of hydrolyzed casein**

ADDITIONS TO BASAL MEDIUM	LENGTH OF FERMENTATION	ACID PRODUCED PER 100 CC. MEDIUM
	days	*cc. 0.1 N*
None	6	28.0
3.0 cc. Neuberg precipitate	6	57.0
3.0 cc. hydrolyzed Neuberg precipitate	6	58.0
1 gram hydrolyzed* casein	7	62.0
0.6 gram diamino acid fraction*	7	55.0
0.6 gram dicarboxylic acid fraction*	7	47.0
0.6 gram mono-amino acid fraction*	7	48.0

Basal medium—1 per cent corn, 1 per cent glucose, 3 cc. Neuberg filtrate fraction per 100 cc.

* The optimum concentration of these fractions was not determined.

The results are given in table 1. The hydrolysis did not inactivate the fraction since the hydrolyzed material supplemented the filtrate fraction just as well as did the unhydrolyzed.

Hydrolyzed casein as the equivalent of the Neuberg precipitate. From the above experiment it seemed probable that the active principle in the Neuberg precipitate was an amino acid. Therefore a mixture of amino acids was prepared by hydrolyzing casein. The hydrolyzed casein was also further separated into four amino acid fractions: least soluble, dicarboxylic, diamino and monoamino (Windus, *et al.* (1931)). The last three fractions, as well as whole hydrolyzed casein, were tested as to their ability to

replace the Neuberg precipitate. The results are given in table 1. It was found that the amino acids from casein completely replaced the Neuberg precipitate of potato. Similarly, each of the three fractions of these amino acids was almost as good as the Neuberg precipitate. The diamino acid fraction seemed to be slightly better than the other fractions. The dicarboxylic and the mono-amino fractions were about equally effective, but as will be shown later, the dicarboxylic fraction may have been slightly toxic in the concentrations used so that better results might have been obtained with a lower concentration. These results indicate rather conclusively that the amino acids of casein can replace the Neuberg precipitate fraction of potato.

Replacement of Neuberg precipitate with various nitrogenous compounds. The nitrogenous compounds were tested in a corn-mash glucose medium containing the optimal concentration of Neuberg filtrate. Since the Neuberg precipitate of potato extract and the casein hydrolyzate both contained some ammonia, the effect of ammonium sulphate was also determined. The results are shown in figure 1. The concentrations were so adjusted that equivalent amounts of total nitrogen were added. Four concentrations were tried, corresponding to 6.0, 18.0, 30.0 and 60.0 mgm. of total nitrogen added to 100 cc. of medium. The results show that ammonium nitrogen was completely effective. The acid production reached the maximum and leveled off at a concentration of 18.0 mgm. of nitrogen per 100 cc. of medium. No toxic effect of a higher concentration was observed, however. Urea nitrogen was practically as effective as ammonium nitrogen. The effect of peptone did not reach a maximum until about 60.0 mgm. of nitrogen were added to 100 cc. It was assumed that the concentration of nitrogen in peptone was about that in protein; *i.e.*, 16 per cent. The only pure compounds containing amino nitrogen which approached ammonia in availability were asparagine and glutamic acid, both of which are acidic in nature. Asparagine was as effective as ammonia, producing the maximum stimulation at the same concentration of nitrogen (18.0 mgm. per 100 cc.). In contrast to ammonia, however, higher concentrations of asparagine seemed to be de-

cidedly toxic. Glutamic acid, at a concentration of 6.0 mgm. N per 100 cc. was as effective as an equivalent concentration of ammonium or asparagine nitrogen. However, its toxic effect became evident at a lower concentration of nitrogen than did that of asparagine. The diamino acids, of which histidine is

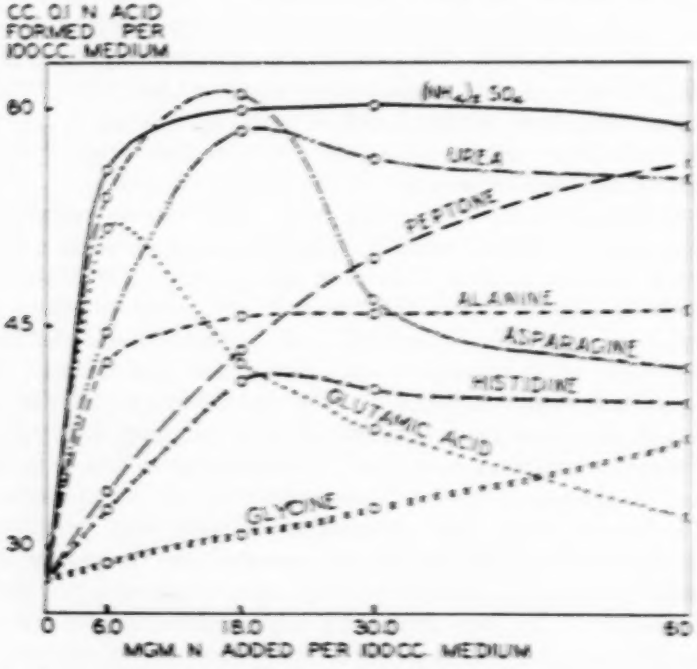


FIG. 1. EFFECT OF VARIOUS NITROGEN COMPOUNDS ON ACID PRODUCTION BY CULTURE 11

(Seven days' fermentation. Basal medium contained 3.0 cc. Neuberg filtrate per 100 cc., 1 per cent corn, and 1 per cent glucose.)

given as an example, did not seem to be very effective, since the acid production was only about half as great at a concentration of 60 mgm. N per 100 cc. as was that with ammonia, urea or asparagine at a concentration of 18 mgm. N per 100 cc. Alanine was slightly better; about two-thirds as good as ammonia at a concentration of 18 mgm. N. However, a higher concentration

of alanine had no further effect. The other mono-amino acids tried (glycine, leucine, tyrosine and cystine) had very little effect at any concentration. Glycine was the least effective.

The complete replacement of the Neuberg precipitate by ammonia and by asparagine, both of which are present in this fraction, indicates that the activity of this fraction can be attributed to these substances.

It should be pointed out that the Neuberg precipitate of potato had no stimulatory action except in the presence of the Neuberg filtrate and the corn factor. Similarly, none of the nitrogenous substances which can replace the precipitate was effective alone. The Neuberg filtrate of potato and the corn factor were essential in making possible the utilization of nitrogen from all of these sources.

Proof of utilization of ammonia. In order to show that ammonium nitrogen was actually available and was utilized by the organisms in the presence of Neuberg filtrate of potato, a direct determination was made of the ammonia utilized in a fermentation. A water extract of corn was used in place of corn meal. This extract was shown (Tatum, Peterson and Fred (1936)) to be equivalent to the corn itself. The medium as made up consisted of corn extract (equivalent to 1 per cent corn), Neuberg filtrate (3 cc. per 100 cc. medium), ammonium sulphate (approximately 0.3 per cent), glucose (2 per cent) and phosphate buffer (0.15 M at pH 7.0). The glucose and phosphate were sterilized separately and added at the time of inoculation. Two flasks were prepared and one inoculated with a washed suspension of bacteria grown in yeast-extract medium. After eight days' incubation in which time the bacteria grew luxuriantly, both flasks were made up to volume to eliminate error because of evaporation and ammonia was determined in aliquot samples of each. The uninoculated flask contained 55.0 cc. of 0.1 N NH_4OH per 100 cc. of medium and the inoculated flask 46.2 cc. The difference, 8.8 cc. is equivalent to a utilization of 12.3 mgm. nitrogen per 100 cc. The data given in figure 1 show that the optimum concentration of ammonium nitrogen is 18 mgm. per 100 cc. of corn mash medium. When the difference in the two experiments

cidedly toxic. Glutamic acid, at a concentration of 6.0 mgm. N per 100 cc. was as effective as an equivalent concentration of ammonium or asparagine nitrogen. However, its toxic effect became evident at a lower concentration of nitrogen than did that of asparagine. The diamino acids, of which histidine is

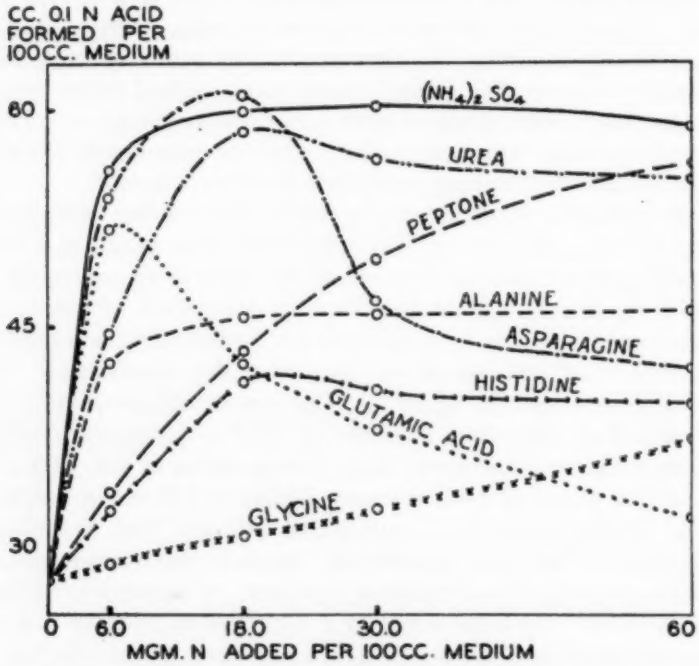


FIG. 1. EFFECT OF VARIOUS NITROGEN COMPOUNDS ON ACID PRODUCTION BY CULTURE 11

(Seven days' fermentation. Basal medium contained 3.0 cc. Neuberg filtrate per 100 cc., 1 per cent corn, and 1 per cent glucose.)

given as an example, did not seem to be very effective, since the acid production was only about half as great at a concentration of 60 mgm. N per 100 cc. as was that with ammonia, urea or asparagine at a concentration of 18 mgm. N per 100 cc. Alanine was slightly better; about two-thirds as good as ammonia at a concentration of 18 mgm. N. However, a higher concentration

of alanine had no further effect. The other mono-amino acids tried (glycine, leucine, tyrosine and cystine) had very little effect at any concentration. Glycine was the least effective.

The complete replacement of the Neuberg precipitate by ammonia and by asparagine, both of which are present in this fraction, indicates that the activity of this fraction can be attributed to these substances.

It should be pointed out that the Neuberg precipitate of potato had no stimulatory action except in the presence of the Neuberg filtrate and the corn factor. Similarly, none of the nitrogenous substances which can replace the precipitate was effective alone. The Neuberg filtrate of potato and the corn factor were essential in making possible the utilization of nitrogen from all of these sources.

Proof of utilization of ammonia. In order to show that ammonium nitrogen was actually available and was utilized by the organisms in the presence of Neuberg filtrate of potato, a direct determination was made of the ammonia utilized in a fermentation. A water extract of corn was used in place of corn meal. This extract was shown (Tatum, Peterson and Fred (1936)) to be equivalent to the corn itself. The medium as made up consisted of corn extract (equivalent to 1 per cent corn), Neuberg filtrate (3 cc. per 100 cc. medium), ammonium sulphate (approximately 0.3 per cent), glucose (2 per cent) and phosphate buffer (0.15 M at pH 7.0). The glucose and phosphate were sterilized separately and added at the time of inoculation. Two flasks were prepared and one inoculated with a washed suspension of bacteria grown in yeast-extract medium. After eight days' incubation in which time the bacteria grew luxuriantly, both flasks were made up to volume to eliminate error because of evaporation and ammonia was determined in aliquot samples of each. The uninoculated flask contained 55.0 cc. of 0.1 N NH_4OH per 100 cc. of medium and the inoculated flask 46.2 cc. The difference, 8.8 cc. is equivalent to a utilization of 12.3 mgm. nitrogen per 100 cc. The data given in figure 1 show that the optimum concentration of ammonium nitrogen is 18 mgm. per 100 cc. of corn mash medium. When the difference in the two experiments

is taken into account, *i.e.*, the use of corn extract and phosphate buffer in place of corn meal, the amount of ammonium nitrogen

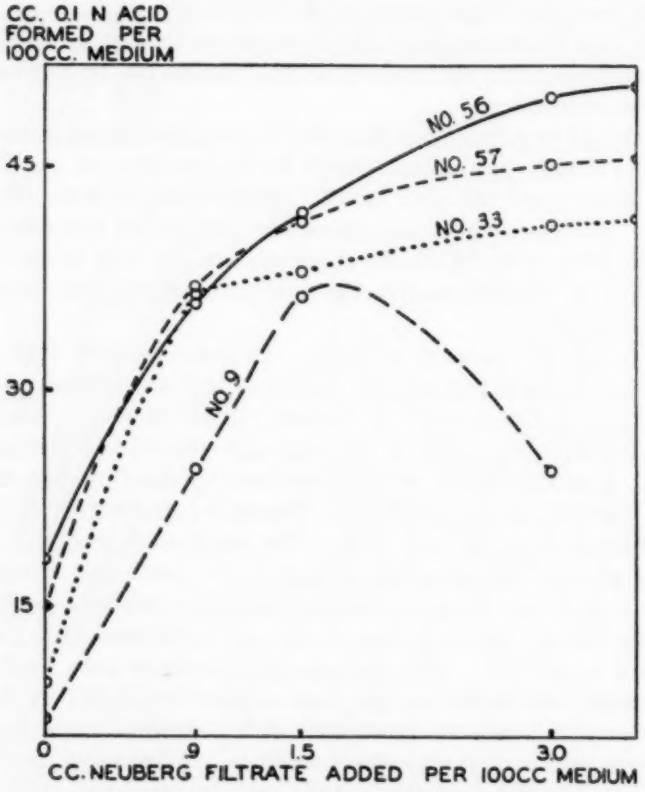


FIG. 2. RESPONSE OF VARIOUS SPECIES OF PROPIONIC ACID BACTERIA TO NEUBERG FILTRATE IN THE PRESENCE OF $(NH_4)_2SO_4$

(Basal medium contained 0.15 per cent $(NH_4)_2SO_4$, 1 per cent corn and 1 per cent glucose.)

actually utilized is in the same range as that found to be the optimum for acid production.

Total nitrogen determinations by the Kjeldahl method showed that 6 mgm. of non-ammonium nitrogen were present per 100 of

medium. It is apparent that the nitrogen utilized was predominantly ammonium nitrogen. In the presence of suitable stimulatory substances the propionic acid bacteria may be able to utilize ammonia as the sole source of nitrogen. Although these bacteria usually have been characterized as requiring complex organic nitrogen compounds for growth, this requirement has not been rigidly proven. Yeast extract and other complex substances used for growing these bacteria may be effective because they contain essential non-nitrogenous growth factors as well as an available source of nitrogen. Purification of the essential factors in Neuberg filtrate and corn extract is now under investigation in an attempt to solve this problem.

Response of other strains to the stimulant. Since *Propionibacterium pentosaceum* no. 11 was found to utilize ammonia in the presence of the Neuberg filtrate, the question arose whether this response to Neuberg filtrate is restricted to the one strain or whether it is a general property of the propionic acid bacteria. *P. zeae* no. 56, *P. pentosaceum* no. 57, *P. Freudenreichii* no. 33 and *P. Shermanii* no. 9, representative of four different groups of propionic acid bacteria which were isolated and studied by Hitchner (1934), were therefore tested (fig. 2). All showed a stimulation of both growth and acid production which was much the same as that given by culture 11. It seems justifiable to conclude that this response is a general characteristic of the propionic acid bacteria, and that all strains may be able to utilize ammonia in the presence of the stimulant.

SUMMARY

The stimulatory effect of the Neuberg precipitate fraction of potato extract on the growth of propionic acid bacteria has been found to be due primarily to the ammonium nitrogen and the asparagine which it contains. Urea, glutamic acid and peptone also can replace this fraction, but a number of amino acids are less effective. All strains tested responded to the stimulant.

Vigorous growth was obtained on a medium containing corn extract, ammonium sulphate, glucose and Neuberg filtrate of potato.

It has been shown that the propionic acid bacteria are able to utilize ammonium nitrogen in the presence of necessary growth factors.

REFERENCES

HITCHNER, E. R. 1934 Jour. Bact., **28**, 473.
TATUM, E. L., PETERSON, W. H., AND FRED, E. B. 1936 Jour. Bact., **32**, 157.
WINDUS, W., CATHERWOOD, F. L., AND ROSE, W. C. 1931 Jour. Biol. Chem., **94**, 173.

BACTERIOLOGICAL APPROACH TO THE PROBLEM OF INTERCONVERSION OF HEXOSES IN ALKALINE SOLUTIONS

A. G. WEDUM

Department of Bacteriology, Northwestern University Medical School, Chicago

Received for publication March 27, 1936

The demonstration by Lobry de Bruyn and van Ekenstein and succeeding workers of the interconversion of hexoses in alkaline solutions has often caused bacteriologists some apprehension. Just how much interconversion does occur in the mildly alkaline culture mediums containing simple sugars that are used in studying the fermentation reactions of bacteria? This possibility may be minimized because the bacteriological incubation periods are too short to permit much alteration of the sugar and because the chemical studies demonstrating interconversion have usually been carried out in much more alkaline solutions than are suitable for culture mediums.

A recent quantitative study (Wedum, 1936) of the fermentation reactions of bacteria which had been cultivated in alkaline sugar mediums for three months incidentally showed that no appreciable interconversion of hexoses had occurred. This was particularly striking in the case of *Bacterium proteus* and *Bacillus anthracis* in mannose mediums. Seven strains of *B. proteus* and 3 of *B. anthracis* were found to ferment glucose and fructose but not mannose. If the interconversion system glucose $\rightleftarrows$ fructose $\rightleftarrows$ mannose were operative, one would expect the organisms gradually to cause an appreciable diminution in the amount of reducing sugar in the medium, but such was not the case. It may be proved that the organisms are capable of metabolizing glucose or fructose during the long incubation period by adding to such old cultures 0.5 per cent glucose or fructose and an acid-indicator.

Consideration of the work of Spoehr and Strain (1929) indicates

that a more direct bacteriological attack upon this problem of interconversion may be undertaken. These authors showed that 33 per cent mannose in the presence of 25 per cent disodium phosphate at 37°C. was converted in the course of 166 days into 32 per cent ketose and 22 per cent aldose other than mannose. Table 1 is compiled from their results and is the basis of the present experiment.

The present work is founded upon the following assumption. If, as is indicated in table 1, 54 per cent of the mannose is converted into what is presumably chiefly glucose and fructose, then it should be possible to demonstrate evidences of fermentation

TABLE 1

Alteration of 33 per cent mannose at 37°C. in 25 per cent disodium phosphate

(From Spoehr and Strain)

TIME	pH	TOTAL REDUCING POWER	PERCENTAGE OF ORIGINAL MANNOSE				
			Hexose other than mannose	Ketose	Aldose	Glucose*	Mannose*
days							
(1 hour)	8.16	85.0	10	0	100	10	90
27	7.74	89.7	24	13	87	11	76
71	7.57	90.2	39	22	78	17	61
166	7.35	89.8	54	32	68	22	46

* Assuming that these are the only aldoses present.

by introducing *B. proteus* or *B. anthracis* with an acid indicator into such a solution. That is, the glucose or fructose should be selectively fermented with production of evident acid and gas while the mannose remains untouched because these bacteria ferment glucose and fructose but not mannose.

METHODS

Preparation of mannose solutions[1]

Solution 1. An aqueous solution of 44 per cent mannose[2] and one of 100 per cent $Na_2HPO_4 \cdot 12H_2O$ were autoclaved separately

[1] These procedures followed those of Spoehr and Strain except that (1) xylene was not added as a preservative, (2) the volume here was 1 cc. per tube instead of 333 cc. in a flask.

[2] C.p. Pfanstiehl. Melting point of phenyl hydrazone 195° to 200°C.

at 15 pounds for 20 minutes and then thoroughly mixed in such proportions as to provide a 33 per cent mannose 25 per cent phosphate solution. One cubic centimeter of the mixture was then placed with aseptic precautions in each of several sterile 12 by 100 mm. test tubes, the sterile cotton stopper replaced, cut short, singed, and a freshly paraffined cork inserted. The pH as determined colorimetrically was 8.4 or above.

Solution 2. 66 per cent mannose and 50 per cent $Na_2HPO_4 \cdot 12H_2O$ were similarly treated to provide a 33 per cent mannose 25 per cent phosphate solution which was similarly distributed. The pH was 8.8 (colorimetric).

Solution 3 contained 33 per cent mannose and 12.5 per cent $Na_2HPO_4 \cdot 12H_2O$. The pH was 8.7.

Solution 4 contained 33 per cent mannose and 25 per cent acid phosphate $NaH_2PO_4 \cdot H_2O$. The pH was 5.2.

The tubes were incubated at 37°C. for 27 or 166 days.

Bacteriological examination of the solutions

At 27 and 166 days certain of the tubes were opened and under aseptic precautions (1) the pH was determined colorimetrically, (2) a loopful of material was transferred to Andrade mannose or maltose agar plates or to Andrade mannose broth tubes as a test for sterility, (3) acid or alkali was added to reduce the buffering effect of the phosphates (0.1 cc. normal H_2SO_4 to the alkaline solutions 1, 2, 3 and 0.5 to 1.0 cc. normal NaOH to the acid solution 4), (4) 3 cc. of a 24 hour 1 per cent Andrade indicator, sugar-free, beef-heart-infusion broth culture of *B. proteus* or *B. Anthracis* was added, (5) cultures were then incubated at 37°C. and observed for acid and gas at intervals of not greater than 12 hours for a period of 48 hours, (6) after 48 hours in order to determine just how much known glucose or fructose would permit the production of evident acid and gas, 0.2 or 0.4 cc. of sterile, (Berkefeld filtered) 10 per cent glucose or fructose was added, (7) cultures were again observed at frequent intervals for acid and gas.

RESULTS

Table 2 indicates that after 166 days incubation at 37°C. the mixture of 33 per cent mannose 25 per cent phosphate gave no indication of the presence of glucose or fructose when examined bacteriologically, although small amounts of added known glucose or fructose caused the formation of evident acid or gas.

TABLE 2

Bacteriological examination of 33 per cent mannose 25 per cent phosphate (solution 1) after 166 days at 37°C.

TUBE	pH	3 CC. 24-HOUR ANDRADE BROTH CULTURE ADDED	C.C. NORMAL H_2SO_4 ADDED	SUBSEQUENT COURSE OF FERMENTATION	KNOWN 10 PER CENT SUGAR SOLUTION ADDED AT 49TH HOUR	SUBSEQUENT COURSE OF FERMENTATION		
						16 hours	24 hours	48 hours
1	7.5	Anthrax 28	0.1	Abundant growth	0.4 cc. fructose	0	sl A	sl A
2	7.5	Proteus 78	0.1	but no acid or gas	0.2 cc. glucose	sl G	AG	AG
3	7.5	Proteus 226	0.1	in 48 hours	0.4 cc. glucose	G	G	
4	7.6	Coli I	0.1	AG	None			

sl = slight; A = acid; G = gas.

TABLE 3

Theoretical concentrations of converted hexoses in the test cultures

	CONCENTRATION OF CONVERTED HEXOSE IN THE TEST CULTURE*
	per cent
If all of the converted 54 per cent mannose were glucose.....	4.3
If all of the converted 54 per cent mannose were fructose.....	4.3
If all of the converted 22 per cent aldose were glucose.....	1.8
If all of the converted 32 per cent ketose were fructose.....	2.6

* One cubic centimeter mannose-phosphate, 3 cc. broth culture, 0.1 cc. normal sulphuric acid.

All sugar solutions before the addition of the bacteria were sterile as evidenced by transfer to plates or broth. Cultures were examined for acid and gas at 4, 8, 18, 24, 30, 42, 48 hours. *Escherichia coli* was included as a control because it ferments glucose, fructose, and also mannose.

Similar results were obtained at 166 days with all of 5 tubes of

solution 2, all of 6 tubes of solution 3, and all of 3 tubes of solution 4. Solutions 2 and 4 were tested with an additional organism, Proteus X 19. The acid or gas present after addition of known glucose or fructose was most marked in tubes of solution 3. Corresponding results had been previously obtained in other tubes tested after 27 days incubation of the 4 mannose-phosphate solutions.

Table 3 shows how much converted hexose, theoretically, should be present in the bacteriological test cultures of table 2. provided the conversion proceeded according to table 1.

TABLE 4

*Known glucose or fructose concentrations showing acid and gas within 48 hours**

MANNOSE-PHOSPHATE SOLUTION	GLUCOSE				FRUCTOSE			
	0.88 per cent tubes showing		0.47 per cent tubes showing		0.88 per cent tubes showing		0.47 per cent tubes showing	
	AG	None	AG	None	AG	None	AG	None
1	2	0	1	0	3	1		
2	5	0	2	0	0	1	0	1
3	2	0	4	0	1	1	2	0
4	2	2	1	0				

AG = acid and/or gas.

* In a culture consisting of 1 cc. mannose-phosphate, 3 cc. Andrade broth culture, 0.1 cc. normal sulphuric acid (sodium hydroxide in solution 4), 0.2 or 0.4 cc. 10 per cent glucose or fructose.

B. anthracis forms no gas.

Table 4 summarizes the experiments designed to determine just how much glucose or fructose was actually necessary in order to permit the production of evident acid and gas in the mannose-phosphate culture mixtures. The hexose concentrations should be compared with those in table 3. The table includes all tests made at the 49th hour (cf., e.g., table 2) in all 4 solutions at both 27 and 166 days with the 3 strains of *B. proteus* and 1 strain of *B. anthracis*. The presence of acid and gas in some tubes and absence in others (e.g., solution 4) merely represents differences in the fermentative potency of various strains of the bacteria.

Tables 3 and 4 emphasize the fact that if either one fifth of the

theoretical amount of converted fructose had been present, or one third of the theoretical glucose, acid and gas formation would have been clearly evident. One might well conclude therefore that either there was no such conversion or that the conversion products were not in a fermentable form.

SUPPLEMENTARY CONTROL EXPERIMENTS

Various experiments were performed to supplement the findings of table 4 regarding the amount of glucose or fructose necessary for *B. proteus* or *B. anthracis* to produce evident acid in the presence of phosphate. These tests consisted in the observation of the action of the bacteria in varying amounts of phosphate, glucose, and fructose with or without a 33 per cent concentration of non-fermentable sugar (lactose), and with or without the addition of small amounts of neutralizing sulphuric acid. It does not seem worth while to record these in detail. In general, they corroborated table 4 in the finding that 0.5 to 1 per cent glucose could practically always be detected within 48 hours by acid formation despite a phosphate concentration of 6 per cent;[3] while about 1 per cent fructose was required under similar conditions.

Other sets of experiments were designed to test further the inability of *B. proteus* and *B. anthracis* to ferment mannose.[4] The methods were much the same as those previously described (Wedum, 1936). In all, 75 cultures were made in beef broth and peptone mediums containing 0.5 per cent mannose and 0.5, 1, 2, or 4 per cent disodium phosphate (pH 7.0 to 7.7), or 1 per cent monosodium phosphate (pH 5.6 to 6.1), or sodium hydroxide or sodium carbonate (pH 7.8 to 8.0). Incubation was for 10 to 13 weeks. Quantitative analyses for reducing sugar were made in 10 weeks or at 1, 4, 10 or 1, 4, 13 weeks. The terminal pH in the alkaline media was usually about 8.4. Uninoculated media and *Bacterium alcaligenes* cultures served as controls. With the exception of one inexplainable and irreproduceable set of 6 cul-

[3] The mannose—25 per cent phosphate solutions were reduced to 6 per cent on the addition of 3 cc. broth culture, 0.1 cc. acid, 0.2 to 0.4 cc. glucose or fructose.

[4] Some of the strains of *B. proteus* and *B. anthracis* in the American Type Culture Collection do ferment mannose however.

tures[5] there was no evidence of clear-cut utilization of mannose by 2 strains of *B. anthracis* and 4 strains of *B. proteus*.

DISCUSSION

The failure to detect glucose or fructose in these mannose-phosphate solutions may be explained by the assumption that the glucose or fructose is present in a non-fermentable form. However, this non-fermentable form is sufficiently close to the ordinary fermentable form so that the chemical manipulations employed in the usual interconversion studies either convert it to or cause it to be identified as fermentable glucose or fructose.

Most interesting investigations could be carried out if additional organisms could be secured which would ferment only one or two of the hexoses, glucose, fructose, mannose. Several of these have been reported but the reports have been based upon the use of acid indicators whereas the frequent absence of evident acid production by such atypical organisms makes necessary the employment of a quantitative method of carbohydrate analysis. An extensive search to find such organisms is at present under way.

SUMMARY

According to Spoehr and Strain, 54 per cent of a 33 per cent solution of mannose in 25 per cent disodium phosphate which has been held at 37°C. for 166 days is converted into other aldoses and ketoses, presumably chiefly glucose and fructose.

When *Bacterium proteus* or *Bacillus anthracis*, which ferment glucose and fructose but not mannose, are introduced into such solutions on the 167th day there is no fermentative evidence of the presence of glucose or fructose.

It is shown that under the conditions of the experiment if either one fifth of the theoretical amount of converted fructose were present, or one third of the theoretical glucose, fermentative evidence of their presence would have been elicited.

[5] No evident acid was produced upon transfer to mannose indicator plates or to mannose indicator broth. Nevertheless the possibility of the presence of slow-fermenting mannose variants must be considered.

It is suggested (1) that the glucose or fructose is present in a non-fermentable form and (2) that this non-fermentable form is sufficiently close to the fermentable form so that the chemical manipulations employed in the usual interconversion studies either convert it to or cause it to be identified as fermentable glucose or fructose.

REFERENCES

SPOEHR, H. A., AND STRAIN, H. H. 1929 Jour. Biol. Chem., **85**: 365.
WEDUM, A. G. 1936 Jour. Infect. Dis., (In press).

PHYSIOLOGICAL STUDIES ON RHIZOBIUM

V. THE EXTENT OF OXIDATION OF CARBONACEOUS MATERIALS[1]

O. R. NEAL[2] AND R. H. WALKER[3]

Iowa State College, Ames, Iowa

Received for publication March 28, 1936

The earlier papers of this series (Walker, Anderson and Brown, 1933; Neal and Walker, 1935) dealt with the growth and respiration of *Rhizobium* under varying environmental conditions. In all cases, the organisms were provided with a sufficient source of energy to permit unhindered respiratory activity throughout the experiments. If only a small amount of the carbonaceous material is used in the substrate, on the other hand, the extent of oxidation of the compound caused by the organisms, may be determined within a reasonably short period. The procedure consists in comparing the volume of oxygen consumed by the culture with the volume that would be required for complete oxidation of the compound to carbon dioxide and water. The extent of oxidation of certain carbonaceous materials which can be effected by *Rhizobium meliloti* and *R. japonicum*, respectively, has been determined by this method and the results are reported in this paper.

METHODS

Warburg's (1926) respiration methods were employed in making continuous measurements of the oxygen consumed and the carbon dioxide produced by the organisms. In preliminary experiments,

[1] Journal Paper J-337, Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 226.

[2] Now with the Soil Conservation Service, in charge of the Soil Erosion Farm at Clarinda, Iowa.

[3] The authors are indebted to Dr. P. E. Brown for the suggestions and criticisms offered in the course of this work and in the preparation of the manuscript.

special Bunsen absorption coefficients were determined experimentally for the absorption of CO_2 in the particular media employed and under the pressures of gas encountered in the later studies. These coefficients were substituted in Warburg's equation and used in calculating the vessel constant for each of the respiration vessels used, without alkali in the inner well.

The following basic medium was employed throughout, K_2HPO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.1 gram; $CaCO_3$, 3.0 grams; distilled water, 1,000 cc. Either sodium nitrate or ammonium chloride was added to the medium to provide 100 p.p.m. of nitrogen in each case. Glucose or other carbohydrate was added in varying minimum amounts as will be described later. All experiments were conducted in duplicate and the results given are averages of two closely agreeing determinations. The inocula were prepared from organisms grown for 3 to 4 days in a glucose-yeast extract liquid medium, then centrifuged and washed twice in a sterile 0.425 per cent NaCl solution. A 1 cc. suspension of washed cells was placed in the respiration vessel which contained 1 cc. of a double concentration of the substrate. The vessels were immediately connected with the manometers and placed on the water bath to attain a temperature equilibrium before being closed to start the experiment. The temperature of the bath was maintained at 28.0°C. $\pm 0.01°$. The manometers were oscillated 90 to 95 times per minute, with an amplitude of approximately 3 cm.

EXPERIMENTAL

Rhizobium meliloti

In the first experiment, *Rhizobium meliloti* was grown in media containing M/540, M/270, and M/180 concentrations of glucose. In order to determine the extent of the endogenous respiration by the organisms of the inocula, a fourth culture vessel was included in the experiment, in which the organisms were cultured as in the other three vessels except that there was no carbohydrate in the medium. Deductions were then made of the quantities of oxygen consumed in the controls from the amounts consumed in

the vessels containing glucose. The corrected amounts of oxygen consumed are shown by the graphs in figure 1.

It is seen that the consumption of oxygen proceeded at about the same rate in each of the media for four hours. At that time there was an abrupt change in the rate of oxygen consumption in the medium, containing M/540 of glucose, and the total amount of oxygen consumed in that culture was not increased appreciably during the remainder of the experiment. At later periods, similar changes occurred in the rate of respiration of the organisms in the other media. The amount of oxygen consumed before these

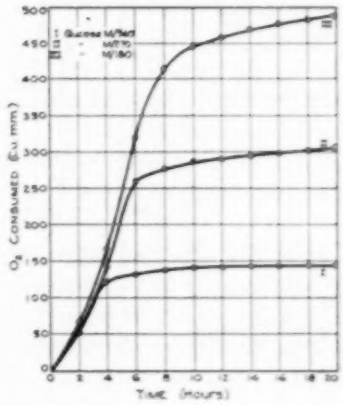


FIG. 1. OXYGEN CONSUMPTION BY RHIZOBIUM MELILOTI IN GLUCOSE MEDIA

changes occurred was almost in direct proportion to the concentration of glucose in the medium.

The amount of oxygen consumed per 2-hour interval, in each of the three media, is shown in figure 2. In each case the rate reached a maximum very quickly, then dropped to a low and fairly constant level. It is apparent that the maximum rate of oxygen consumption was a function of the concentration of glucose in the medium.

Upon calculation of the theoretical amounts of oxygen required to oxidize completely the glucose in each of the media it is found that the M/540, M/270, and M/180 concentrations of the sugar

special Bunsen absorption coefficients were determined experimentally for the absorption of CO_2 in the particular media employed and under the pressures of gas encountered in the later studies. These coefficients were substituted in Warburg's equation and used in calculating the vessel constant for each of the respiration vessels used, without alkali in the inner well.

The following basic medium was employed throughout, K_2HPO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.1 gram; $CaCO_3$, 3.0 grams; distilled water, 1,000 cc. Either sodium nitrate or ammonium chloride was added to the medium to provide 100 p.p.m. of nitrogen in each case. Glucose or other carbohydrate was added in varying minimum amounts as will be described later. All experiments were conducted in duplicate and the results given are averages of two closely agreeing determinations. The inocula were prepared from organisms grown for 3 to 4 days in a glucose-yeast extract liquid medium, then centrifuged and washed twice in a sterile 0.425 per cent NaCl solution. A 1 cc. suspension of washed cells was placed in the respiration vessel which contained 1 cc. of a double concentration of the substrate. The vessels were immediately connected with the manometers and placed on the water bath to attain a temperature equilibrium before being closed to start the experiment. The temperature of the bath was maintained at 28.0°C. ±0.01°. The manometers were oscillated 90 to 95 times per minute, with an amplitude of approximately 3 cm.

EXPERIMENTAL

Rhizobium meliloti

In the first experiment, *Rhizobium meliloti* was grown in media containing M/540, M/270, and M/180 concentrations of glucose. In order to determine the extent of the endogenous respiration by the organisms of the inocula, a fourth culture vessel was included in the experiment, in which the organisms were cultured as in the other three vessels except that there was no carbohydrate in the medium. Deductions were then made of the quantities of oxygen consumed in the controls from the amounts consumed in

the vessels containing glucose. The corrected amounts of oxygen consumed are shown by the graphs in figure 1.

It is seen that the consumption of oxygen proceeded at about the same rate in each of the media for four hours. At that time there was an abrupt change in the rate of oxygen consumption in the medium, containing M/540 of glucose, and the total amount of oxygen consumed in that culture was not increased appreciably during the remainder of the experiment. At later periods, similar changes occurred in the rate of respiration of the organisms in the other media. The amount of oxygen consumed before these

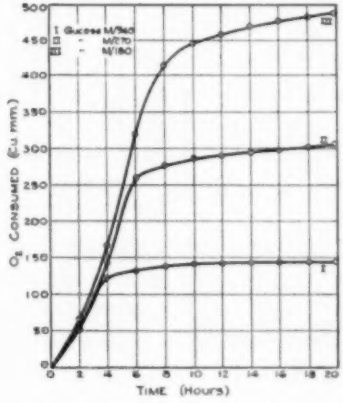


FIG. 1. OXYGEN CONSUMPTION BY RHIZOBIUM MELILOTI IN GLUCOSE MEDIA

changes occurred was almost in direct proportion to the concentration of glucose in the medium.

The amount of oxygen consumed per 2-hour interval, in each of the three media, is shown in figure 2. In each case the rate reached a maximum very quickly, then dropped to a low and fairly constant level. It is apparent that the maximum rate of oxygen consumption was a function of the concentration of glucose in the medium.

Upon calculation of the theoretical amounts of oxygen required to oxidize completely the glucose in each of the media it is found that the M/540, M/270, and M/180 concentrations of the sugar

would require, respectively, 497, 994, and 1491 c. mm. of oxygen. This calculation necessarily assumes that oxygen is the sole hydrogen acceptor in the dissimilation process. The actual amounts of oxygen consumed by the organisms during a 24-hour period in the three media, in increasing order of the glucose concentration, were 143, 306, and 488 c. mm. These values represent approximately 30 per cent of the theoretical amount of oxygen necessary for complete oxidation of the glucose.

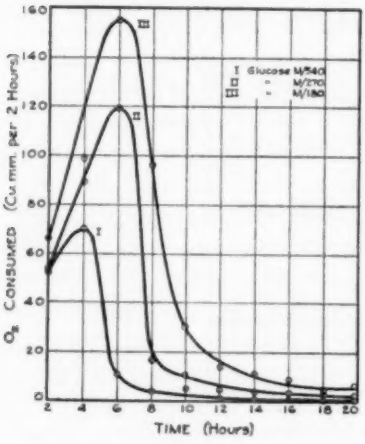


FIG. 2

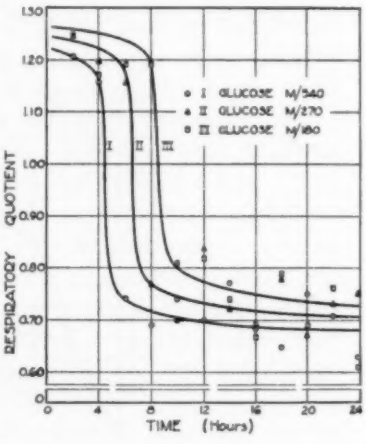


FIG. 3

FIG. 2. RATE OF OXYGEN CONSUMPTION BY RHIZOBIUM MELILOTI IN GLUCOSE MEDIA

FIG. 3. RESPIRATORY QUOTIENT OF RHIZOBIUM MELILOTI IN GLUCOSE MEDIA

It is possible that the organisms utilized only a fraction of the glucose, oxidizing it completely to carbon dioxide and water. It seems more probable, however, that all of the glucose was partially dissimilated with the production of intermediate or end products other than carbon dioxide. The abrupt drop in the rate of respiration as shown by the curves in figure 2 and which was observed in every experiment, would only be expected in a case where a drastic change occurred in the medium, such as a change in pH, temperature, aeration, or depletion of the food supply.

Inasmuch as the temperature, aeration, and pH of the media were controlled it is logical to assume that there was a depletion of food supply at the point of deflection in the rate curve. Furthermore, when the total amounts of oxygen consumed by the organisms in the different concentrations of glucose are computed, as previously described, in percentage of the total amount of oxygen required for complete oxidation of the entire amount of sugar, it is found that the values obtained up to the inflection point in the curve in the different experiments agree rather closely. These values were 32.7, 30.7, and 28.9 per cent, respectively, for the M/180, M/270, and M/540 concentrations of glucose.

The oxidation process is apparently stopped when it reaches a certain stage. Obviously this would not be the case if the organisms oxidized the dissimilated glucose completely. The data indicate, however, that the molecules of glucose are oxidized to a certain stage, whereupon the oxidation stops, leaving the residual material as intermediate or end products which are not utilized by the organism.

The changes in respiratory quotient that occurred during the experiment substantiate this viewpoint. From the time the organisms were introduced into the various media until the rate of oxidation decreased abruptly, the respiratory quotient was considerably above unity in all cases and ranged from 1.16 to 1.26.[4] This is shown by the curves in figure 3. Furthermore, it may be noted that there was an abrupt decrease in respiratory quotient from above unity to 0.8 or lower and that this occurred simultaneously with the decrease in rate of oxygen consumption.

This may be interpreted as indicating that the organisms were obtaining their energy for growth from the oxidation of glucose in the early part of the experiment. When the dissimilation of glucose had been carried as far as possible under the imposed conditions, it was no longer feasible for the organisms to obtain energy from that source, and as a result oxygen consumption was materially reduced. Furthermore, in order that the organisms

[4] A more complete report on the significance of deviations of respiratory quotient from unity during the various growth phases will be presented for publication in the near future.

might continue to live, it became necessary to utilize the energy stored in the material of their cells, the proteins and fats. When these materials are burned in the air, their oxygen quotients are approximately 0.8 and 0.71, respectively. Thus, the low respiratory quotients of the organisms in the latter part of the experiment indicate that the organisms were oxidizing the stored proteins and fats for subsistence, and that they were not able to attack the intermediate or end products of the primary glucose oxidation, if there were any other outside the bacterial cell material itself. Undoubtedly, a large proportion of the original glucose not accounted for in the oxygen produced was transformed into cell material, and it is probably this material that was being oxidized, although slowly, in the latter part of the experiment.

These results and interpretations are in strict agreement with those of Burk and Horner (1936), who concluded that the extracellular ammonia produced by *Azotobacter* is, in all probability, entirely derived from decomposition of normal cell nitrogen and not by direct synthesis from free nitrogen. They found that the formation of ammonia by *Azotobacter* never commenced until the concentration of utilizable organic substrate had reached a very low level of about 0.03 to 0.01 per cent so that the substrate had essentially disappeared. In the absence of a suitable substrate the *Azotobacter* carried on endogenous respiration by the oxidation of the stored proteins of the cell protoplasm and the excess nitrogen was given off in the form of ammonia as in the ammonification of other proteinaceous materials by microörganisms. Undoubtedly the same process occurred in the present experiments with *Rhizobium*, although no tests were made for the ammonia produced. The experiments reported here were completed before the appearance of the paper by Burk and Horner (1936).

It is evident, therefore, that there was a complete change in the metabolism of the organisms which occurred at a definite time, and this, apparently, was determined by the disappearance of glucose from the substrate.

In a second experiment using mannitol as the test substrate, results very similar to those with glucose were obtained. The rate of oxygen consumption, as shown in figure 4, increased rapidly

to a maximum early in the experimental period, then decreased abruptly to approximately the same level as that of the control. In this experiment the respiratory quotient was observed to range from 0.98 to 1.05 in the different media during the period of rapid oxygen consumption. Simultaneously with the decrease in rate of oxygen consumption there was an abrupt decrease in respiratory quotient to approximately 0.8 or lower, just as was the case when the organisms were grown on a glucose substrate. This

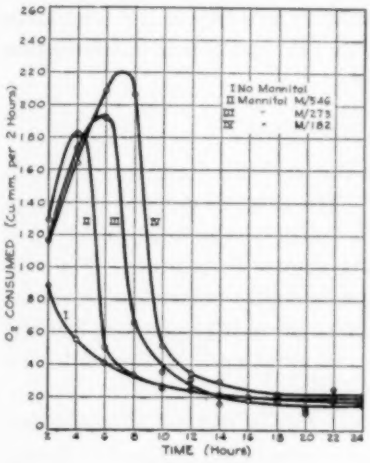


FIG. 4

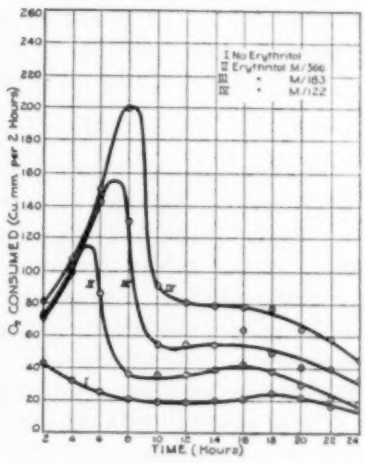


FIG. 5

FIG. 4. RATE OF OXYGEN CONSUMPTION BY RHIZOBIUM MELILOTI IN MANNITOL MEDIA

FIG. 5. RATE OF OXYGEN CONSUMPTION BY RHIZOBIUM MELILOTI IN ERYTHRITOL MEDIA

again indicates a change of metabolism from one of mannitol dissimilation to one of oxidation of stored proteins and fats. The maximum rate of respiration was apparently a function of the concentration of the material supplying energy and of time. The extent of oxidation of the mannitol, as determined by the total consumption of oxygen, will be discussed later.

In erythritol ammonium-chloride media the rate of oxygen consumption by the alfalfa organism as shown in figure 5, again rose

rapidly to a maximum, then decreased rather abruptly. The respiratory quotient decreased abruptly and simultaneously with the decrease in rate of oxygen consumption. The rate of respiration, however, did not drop to approximately the same level as that of the control, as in the previous cases, but continued at a considerably higher level throughout the experiment. Similarly the respiratory quotient did not drop to as low a value in this experiment and after the drop it varied between approximately 0.80 and 0.90. Apparently, some constituent of the erythritol or some product of the early respiratory activity of the organisms was sufficiently available to permit this continued consumption of oxygen for a considerable period.

Rhizobium japonicum

The dissimilation of glucose by the soybean organism proceeds in a somewhat different manner. The rates of oxygen consumption by this organism when cultured in media containing $\frac{1}{3}$, $\frac{2}{3}$, and 1 gram per liter of glucose, are shown in figure 6. The rate increased slowly to a maximum considerably below that attained by *Rhizobium meliloti*, then there was an abrupt change; but the magnitude of the change was not as great as that shown in the experiments with the alfalfa organism.

Simultaneously with the change in rate of oxygen consumption there was a decrease in the respiratory quotient; during the period of maximum rate of oxygen consumption the quotient varied from 1.10 to 1.20 in the various media, and when the rate of oxygen consumed decreased, the quotient fell to approximately 1.00 or below, ranging from 0.85 to 0.95 in most cases. In this connection *R. japonicum* differed somewhat from *R. meliloti* in that the respiratory quotient in cultures did not drop as low after the carbohydrate dissimilation had ceased. This may be explained, at least partially, by the fact that the soybean organism grew much more slowly on the glucose substrate than did the alfalfa organism, and the rate of oxygen consumption was never as high under similar conditions. The difference in rate of growth may be attributed to the fact that this carbohydrate is less easily attacked by this organism, or perhaps to a difference between the

two organisms in redox potential requirements. These two possibilities may be related to the conditions under which these experiments were conducted. It may be possible also that the dissimilation of glucose is brought about in a somewhat different manner under the conditions imposed here, but this hardly seems logical.

It is important to note, however, that in spite of the difference in rate of oxygen consumption by the two organisms, there oc-

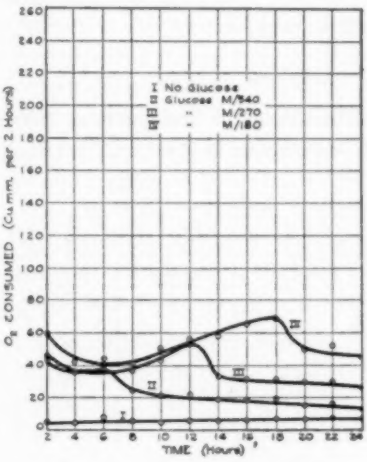


FIG. 6

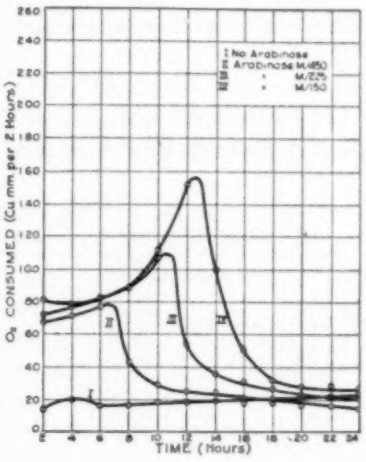


FIG. 7

FIG. 6. RATE OF OXYGEN CONSUMPTION BY RHIZOBIUM JAPONICUM IN GLUCOSE MEDIA

FIG. 7. RATE OF OXYGEN CONSUMPTION BY RHIZOBIUM JAPONICUM IN ARABINOSE MEDIA

curred a similar decrease in rate at a definite stage in the dissimilation process, and that this change in rate was accompanied by a similar change in respiratory quotient.

In arabinose media the rates of oxygen consumption by this organism, as shown in figure 7, were very similar to those exhibited by *R. meliloti* in media containing the various carbon compounds. The maximum rate attained in this experiment was practically the same as that reached by *R. meliloti* in a glucose

medium. The relatively high rate of oxygen consumption shown by *R. japonicum* is of particular interest in view of the fact that the soybean organisms are rather commonly considered as growing slowly. It seems that under favorable conditions, the rate of their activity in dissimilating carbonaceous materials might approach or even equal that of the more rapidly growing species of *Rhizobium*.

It is also significant that in this experiment the changes in rates of oxygen consumption and respiratory quotient were very similar to those found in the earlier experiments and were as marked as those observed in experiments with *R. meliloti*.

The extent of oxidation

The extent of oxidation or of utilization of the various compounds by both *R. meliloti* and *R. japonicum* is shown in table 1.

TABLE 1

Percentage oxidation of various carbonaceous materials by rhizobium

ENERGY SOURCE	½ GRAM PER LITER	¾ GRAM PER LITER	1 GRAM PER LITER
Rhizobium meliloti:			
Glucose	31.3	32.6	34.2
Mannitol	32.7	30.5	34.6
Erythritol	49.4	51.7	48.2
Rhizobium japonicum:			
Glucose	42.6	36.2	37.4
Arabinose	37.6	39.2	39.7

In the discussion of the first experiment, the agreement between the values obtained for different concentrations of the same carbon compound was pointed out in support of the hypothesis that the abrupt change which occurs in the rate of oxygen consumption is due to a change in the food supply of the organisms. At that point the first stage in the dissimilation process is reached, and it is probable that all of the original carbohydrate as such has been utilized, but that a comparatively large proportion of the carbonaceous material has not yet been completely oxidized to carbon dioxide and water.

Neither the mechanism of the dissimilation process nor the

nature of the intermediate and end products remaining in the media have been investigated in this work. At present there is little evidence to permit answering these questions.

Anderson, Peterson, and Fred (1928) demonstrated the production of pyruvic acid by two strains of *Rhizobium meliloti* grown in xylose, glucose, lactose, sucrose, and mannitol media.

Fred, Baldwin and McCoy (1932) reported that only a limited growth with the production of a slightly alkaline reaction was secured with certain cultures of *Rhizobium* on a calcium pyruvate medium. Normally, this compound is thought to be very readily broken down by bacterial action.

Georgi and Wilson (1933) concluded that most of the glucose carbon not appearing as carbon dioxide was transformed into gum. Hopkins, Peterson and Fred (1930) found this gum to consist largely of glucuronic acid.

In anaerobic cultures of the root nodule bacteria, Virtanen, Nordlund, and Holle (1934) found butyric acid produced as an end product. It seems doubtful, however, whether this type of fermentation would occur in aerobic cultures.

It is possible that all, or a part of, the sugar not accounted for by the consumption of oxygen was used in the formation of new cell tissue. This viewpoint is substantiated by the character of the change in respiratory quotient from one of carbohydrate metabolism to one of protein and fat metabolism which occurs simultaneously with the abrupt decrease in rate of oxygen consumption when a definite proportion of total oxygen required for complete oxidation has been consumed.

SUMMARY

Rhizobium meliloti and *Rhizobium japonicum* were cultured in media containing comparatively small quantities of various carbonaceous materials and the oxygen consumed and carbon dioxide produced were measured at regular intervals during a 24-hour period. The rate of oxygen consumption increased consistently until approximately one-third of the theoretical amount required for complete oxidation of the carbohydrate was consumed by the organisms. At that stage in the dissimilation process, there was

an abrupt decrease in rate of oxygen consumption and also in the respiratory quotient of the organisms. This was interpreted as a change from carbohydrate metabolism to one of protein or fat metabolism.

Although the character of the dissimilation process and the intermediate and end products have not been investigated, it seems possible that a large proportion of the carbohydrate not accounted for by the consumption of oxygen was used in the formation of new cell tissue.

Rhizobium japonicum, which is usually looked upon as a slow growing organism, consumed oxygen, or grew, much more rapidly on a substrate containing arabinose than on one containing glucose, and the rate of growth on this medium compared favorably with that of *Rhizobium meliloti* on substrates containing glucose or mannitol.

REFERENCES

ANDERSON, J. A., PETERSON, W. H. AND FRED, E. B. 1928 The production of pyruvic acid by certain nodule bacteria of the Leguminosae. Soil Sci., **25**: 123–131.

BURK, DEAN, AND HORNER, C. KENNETH 1936 The origin and significance of ammonia formed by Azotobacter. Soil Sci., **41**: 81–122.

FRED, E. B., BALDWIN, I. L., AND MCCOY, E. 1932 Root nodule bacteria and leguminous plants. Univ. of Wisc. Studies in Sci., No. 5.

GEORGI, C. E., AND WILSON, P. W. 1933 The influence of the tension of oxygen on the respiration of rhizobia. Arch. Mikrobiol., **4**: 543–564.

HOPKINS, E. W. PETERSON, W. H., AND FRED, E. B. 1930 Composition of the gum produced by root nodule bacteria. Jour. Amer. Chem. Soc., **52**: 3659–3668.

NEAL, O. R., AND WALKER, R. H. 1935 Physiological studies on Rhizobium. IV. Utilization of carbonaceous materials. Jour. Bact., **30**: 173–187.

VIRTANEN, A. J., NORDLUND, M., AND HOLLO, E. 1934 Fermentation of sugar by the root nodule bacteria. Biochem. Jour., **28**: 796–802.

WALKER, R. H., ANDERSON, D. A., AND BROWN, P. E. 1933 Physiological studies on Rhizobium. I. The effect of nitrogen source on oxygen consumption by *Rhizobium leguminosarum* Frank. Soil Sci., **37**: 387–402.

WARBURG, O. 1926 Stoffwechsel der Tumoren. Julius Springer, Berlin.

BACTERIOPHAGY OF BACILLUS MYCOIDES WITH REFERENCE TO EFFECT ON DISSOCIATION AND TRANSMISSION BY SPORES

I. M. LEWIS AND GORDON WORLEY

The University of Texas, Department of Botany and Bacteriology

Received for publication March 30, 1936

Because of its unique colony form *Bacillus mycoides* has been a favorite species for the study of dissociation and life cycles. The mutually confirmatory reports of Nyberg (1929), Lewis (1932) and den Dooren de Jong (1933) have shown that this species is characterized by marked colonial polymorphism and that the sequence of events in dissociation is readily followed in cultures. So far as we are aware no complete study has been made as to the effect of bacteriophage on dissociation in *B. mycoides*. Since marked effects have been reported for other species it seemed that a detailed study of dissociation of *B. mycoides* in the presence of a bacteriophage should afford results of interest and value.

The strains of *B. mycoides* used in a previous investigation of this species, (Lewis (1932)) have been employed in the present study. The bacteriophage was isolated from domestic sewage by a slight modification of the method described by Keller (1931).

Difficulty was encountered in obtaining a high-titer bacteriophage for the original type strains but not for variants. Although numerous feeding and filtration experiments were carried out, the titer was never more than about 10^{-5} for the original while a titer of 10^{-8} was readily obtained for the variants. Secondary growth occurred in all broth cultures with age. Plaques which developed in nutrient 1 per cent agar were rather large and corresponded roughly with the titer obtained in nutrient broth. When spread on the surface of inoculated agar plates the variant strains showed complete clearing, in which some secondary growth could

be noted. Plates of the original strains were not completely cleared, although the lytic action was pronounced. A thin glassy, veil-like growth persisted.

EFFECT OF BACTERIOPHAGE ON DISSOCIATION AND REVERSION

The strains selected for experiments as to the effect of the lytic agent on dissociation and reversion had been in culture for several years. At no time have any of the strains shown spontaneous lysis and filtrates from broth cultures had no lytic effect on other strains of *Bacillus mycoides*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus anthracis* or on various unidentified strains of the subtilis group. Similarly, variant strains isolated from broth cultures have not shown lysis nor the presence of a lytic principle in broth cultures. It is believed, therefore, that all strains included in the experiments were pure.

Since dissociation of *B. mycoides* occurs readily in flask cultures of beef-extract broth this method was followed in testing the effect of bacteriophage on dissociation. Each flask containing 300 cc. of medium received 1 cc. of a young broth-culture and 3 cc. of lytic filtrate. Control flasks without filtrate were included. The cultures were incubated at 30°C., which is the optimum temperature for dissociation under normal conditions.

Plating from the flasks brought out the significant fact that dissociation occurred much more promptly in the absence of bacteriophage than in its presence. Variant strains could be obtained from the control cultures within 5 days while the cultures containing bacteriophage showed no variants until a period of about three weeks had elapsed. We have not been able to obtain any variants from cultures containing bacteriophage which could not be obtained from control flasks. Since the variants are more susceptible to the lytic principle than original strains it appears that these results are due to selective action.

Previous experiments have shown that smooth strains of *B. mycoides* do not revert to the original thread-like type when cultivated under ordinary conditions. Cultures of ten relatively smooth variants were tested as to the effect of bacteriophage on reversion. Flasks containing 300 cc. of beef extract broth re-

ceived 1 cc. from a young broth culture and 3 cc. of active lytic filtrate. The cultures became clear, with subsequent clouding. Plating from these flasks revealed that reversion to the original type had occurred in 2 cases while no reversion could be discovered in control flasks without bacteriophage. Again, the action appears to be selective.

FILTRATION OF PHAGE-LYSED CULTURES

Various workers have reported the presence of filtrable forms in phage-lysed cultures of different species. A filtrable phase of *B. mycoides* has also been reported by Oesterle and Stahl (1930) in strains presumably free from bacteriophage. On the other hand, a filtrable phase could not be obtained by Lewis (1932) or by den Dooren de Jong (1933) from normal broth cultures.

Our results with filtration experiments on phage-lysed cultures of *B. mycoides*, both original and variant strains, have been consistently negative. Filtrates of lysed cultures of different ages from a few hours to several days or weeks, have shown no growth after prolonged incubation, and all attempts to isolate organisms from them by inoculation into broth or plating by Hauduroy's method have failed to produce visible growth.

TRANSMISSION OF BACTERIOPHAGE BY SPORES

Filtrates of phage-lysed cultures of *B. mycoides* were inactivated at 75°C. in ten minutes. Spore suspensions from partially lysed agar slant cultures gave rise to lytic cultures after heating at 90°C. for ten minutes. Similar suspensions heated in the same manner and then filtered failed to yield lytic filtrates.

Whether bacteriophage survives as such within the spore as suggested by Cowles (1931) and Vedder (1932) or is generated *de novo* upon germination, as advocated by den Dooren de Jong, 1930, we have not been able to determine with certainty. Repeated efforts have been made to obtain a lytic filtrate from heated macerated spores of cultures containing bacteriophage. Grinding the spores with sand for approximately one hour followed by extraction at 15,000 pounds pressure and filtration failed to produce a lytic filtrate. The same result was obtained

by grinding with dry ice. The evidence, so far as it goes, supports the theory of induction.

SUMMARY

Strains of *Bacillus mycoides* cultivated in broth containing homologous bacteriophage produce the same variants as in phage-free medium. The variants appear more promptly in the absence of bacteriophage than in its presence. Reversion from the smooth variant to the typical thread-like colony occurred in two of the test strains but not in eight additional strains. Since variants are more sensitive than the original type the action is regarded as selective. A filtrable phase could not be demonstrated.

Suspensions from partially lysed agar cultures could not be rendered free from bacteriophage by heating. Filtrates from heated spore suspensions were non-lytic but cultures developing from heated suspensions became lytic. Attempts to recover intracellular phage by maceration and filtration of heated spore suspensions were not successful.

REFERENCES

Cowles, Philip B. 1931 Jour. Bact., **22**, 119.

den Dooren de Jong, L. E. 1930 Proc. Kon. Akad. Wetensch. te Amsterdam, **33**, 1–5 and **35**, 4–11.

den Dooren de Jong, L. E. 1933 Archiv für Microbiologie, **4**, 36–53.

Keller, M. 1931 Jour. Bact., **22**, 199–208.

Lewis, I. M. 1932 Jour. Bact., **24**, 381–418.

Nyberg, C. 1929 Acta Soc. Med. Fenn., **12**, 3–18.

Oesterle, P. and Stahl, C. A. 1930 Centralbl. f. Bakt., etc., II Abt., **79**, 1–25.

Vedder, A. 1932 Centralblt. f. Bakt., etc., I Abt., Orig., **125**, 111.

SPECIFICITY OF THE DYE IN THE CRYSTAL-VIOLET AGAR REACTION OF STAPHYLOCOCCI

GEORGE H. CHAPMAN

Clinical Research Laboratory, 604 Fifth Avenue, New York, New York

Received for publication April 2, 1936

In a previous paper (Chapman and Berens, 1935), it was shown that staphylococci produce orange, violet or white growths on crystal-violet agar. Because no satisfactory explanation of these color changes has been offered, experiments were undertaken in an effort to learn more about the chemistry of the reaction. Attempts to discover the identity of the active molecule of the dye will be described in the present paper.

The crystal-violet agar had the following composition:

Agar	15 grams
Proteose peptone, Difco	5 grams
Meat extract	3 grams
Crystal-violet, Commission Certified 0.1 per cent (based on the dye content and not on crude weight)	3.3 cc.
Water to make	1,000 cc.
pH	6.8±

The medium is quite inhibitive for staphylococci, making it necessary to inoculate heavily to obtain growth. For this reason, it is suitable only for the study of pure cultures.

Crystal-violet agar prepared from a solution of crystal-violet several weeks old had a rose color, was considerably paler than that prepared from fresh solutions, and gave no sharp color differentiation of staphylococcal growths. Many strains which gave pale violet growths on a medium prepared from this solution gave white growths with violet borders on media prepared from fresh solutions.

A freshly prepared solution of Commission Certified crystal-violet gave best results when added to the agar base to make

TABLE 1

Dyes which, when added to proteose lactose agar, did not give color changes with staphylococci similar to those obtained with crystal-violet

Nitro compounds	Martius yellow, naphthol yellow S
Azo dyes	Acid yellow, chrysoidin Y, chrysoidin R, orange G (LO-3)*, Bordeaux red, Janus green B (NJ-2), methyl orange (NN-4), orange II (NOB-2), amaranth, Sudan III (LY-2), Biebrich scarlet, Bismarck brown (LN-1), Congo red (LQ-4), benzopurpurin 4B, trypan blue, orange I
Oxyquinones	Alizarin, alizarin red S, alizarin blue S, alizarin yellow R
Quinone-imides:	
Thiazins	Thionin (NT-4), azure A, azure B, azure C, azure II, methylene blue (LA-5), methylene violet (2 samples), methylene green, toluidine blue O (NU-1), new methylene blue N
Oxazins	Brilliant cresyl blue (NV-10), gallein, gallocyanin, Nile blue sulfate, cresyl violet (NW-3)
Azins	Neutral red (NX-3), neutral violet, safranin O (LS-4), phenosafranin, nigrosin (2 samples)
Phenyl methanes:	
Diphenyl methanes	Auramin
Triphenyl methanes:	
Diamino	Malachite green (LMg-1), brilliant green (NBg-3), light green SF (NL-3), fast green FCF
Triamino	Basic fuchsin (LF-2), pararosanilin, rosanilin (2 samples), rosanilin HCl, new fuchsin, methyl green (NG-3), acid violet, iodine-green, spirit blue AS, acid blue WS, methyl-blue, methyl violet (LMv-1 and 4 other samples), crystal-violet (NC-9), Hoffman violet (2 samples), ethyl-violet and dahlia
Hydroxy	Aurin (rosolic acid) (2 samples), corallin red (2 samples), corallin yellow (2 samples)
Xanthines:	
Pyronins	Pyronin G
Rhodamins	Rhodamin B
Fluoranes	Fluorescein (acid), fluorescein (sodium salt), eosin Y WS (LE-8), methyl eosin AS, ethyl eosin AS, eosin B (NEB-4), eosin B AS, erythrosin Y, erythrosin B (NEB-4), phloxine B, (NPh-4), rose bengal
Sulphonephthaleins	Thymol blue, cresol red, bromphenol blue, brom-thymol blue
Acridines	Acriflavine, proflavine, neutral acriflavine
Natural dyes	Hematoxylin, hematein, orcein, cochineal, indigo carmine, indigo, borax carmine, brazilin, carmine, carminic acid

* The figures in parentheses refer to the certification number of the sample.

dilutions of between 1:300,000 and 1:350,000. Best color differentiation was obtained after 36 hours incubation.

COMPARATIVE TESTS WITH OTHER DYES

Expecting that the active molecule of crystal-violet was a simple one and that it would be found in dyes containing similar chemical groups, a number of such compounds were tested. The results were negative. Therefore, an attempt was made to determine the active chemical group by testing 108 dyes, representing each of the different groups of biological dyes (table 1). Because the optimum dilution of crystal-violet was critical, it was expected that the optimum dilution of other dyes having similar differential properties would also be critical. Therefore, enough of each dye was added to the agar base to make dilutions of 1:10,000, 1:100,000, 1:250,000 and 1:500,000. Each dye agar was inoculated heavily with 10 strains of staphylococci which were selected because of their different hemolytic, coagulating and crystal-violet agar reactions. After 36 hours incubation, the colors of the growths were compared with those which had been produced on crystal-violet agar.

Inconclusive results were obtained with most of the dyes, any color changes being associated with *albus-aureus* differences, i.e., related to pigment. However, significant color changes were noted with the following dyes: 5 samples of methyl-violet, 1 of crystal-violet and 1 of Hoffman violet which gave results similar to those obtained with the original sample of crystal-violet; and bromthymol blue which gave color changes parallel to, but chemically different from, those produced on crystal-violet agar. The results with bromthymol blue agars depended upon the indicator property of the dye.

STUDIES OF METHYL-VIOLET AND RELATED VIOLET DYES

These results suggested that the active molecule of the crystal-violet agar reaction is contained in crystal- and methyl-violet and some samples of Hoffman violet. Therefore, tests were made with a larger series of violets. Because of the close relationship between Hoffman and ethyl-violets and, because one dye was

TABLE 1

Dyes which, when added to proteose lactose agar, did not give color changes with staphylococci similar to those obtained with crystal-violet

Nitro compounds	Martius yellow, naphthol yellow S
Azo dyes	Acid yellow, chrysoidin Y, chrysoidin R, orange G (LO-3)*, Bordeaux red, Janus green B (NJ-2), methyl orange (NN-4), orange II (NOB-2), amaranth, Sudan III (LY-2), Biebrich scarlet, Bismarck brown (LN-1), Congo red (LQ-4), benzopurpurin 4B, trypan blue, orange I
Oxyquinones	Alizarin, alizarin red S, alizarin blue S, alizarin yellow R
Quinone-imides:	
Thiazins	Thionin (NT-4), azure A, azure B, azure C, azure II, methylene blue (LA-5), methylene violet (2 samples), methylene green, toluidine blue O (NU-1), new methylene blue N
Ozazins	Brilliant cresyl blue (NV-10), gallein, gallocyanin, Nile blue sulfate, cresyl violet (NW-3)
Azins	Neutral red (NX-3), neutral violet, safranin O (LS-4), phenosafranin, nigrosin (2 samples)
Phenyl methanes:	
Diphenyl methanes	Auramin
Triphenyl methanes:	
Diamino	Malachite green (LMg-1), brilliant green (NBg-3), light green SF (NL-3), fast green FCF
Triamino	Basic fuchsin (LF-2), pararosanilin, rosanilin (2 samples), rosanilin HCl, new fuchsin, methyl green (NG-3), acid violet, iodine-green, spirit blue AS, acid blue WS, methyl-blue, methyl violet (LMv-1 and 4 other samples), crystal-violet (NC-9), Hoffman violet (2 samples), ethyl-violet and dahlia
Hydroxy	Aurin (rosolic acid) (2 samples), corallin red (2 samples), corallin yellow (2 samples)
Xanthines:	
Pyronins	Pyronin G
Rhodamins	Rhodamin B
Fluoranes	Fluorescein (acid), fluorescein (sodium salt), eosin Y WS (LE-8), methyl eosin AS, ethyl eosin AS, eosin B (NEB-4), eosin B AS, erythrosin Y, erythrosin B (NEB-4), phloxine B, (NPh-4), rose bengal
Sulphonephthaleins	Thymol blue, cresol red, bromphenol blue, bromthymol blue
Acridines	Acriflavine, proflavine, neutral acriflavine
Natural dyes	Hematoxylin, hematein, orcein, cochineal, indigo carmine, indigo, borax carmine, brazilin, carmine, carminic acid

* The figures in parentheses refer to the certification number of the sample.

dilutions of between 1:300,000 and 1:350,000. Best color differentiation was obtained after 36 hours incubation.

COMPARATIVE TESTS WITH OTHER DYES

Expecting that the active molecule of crystal-violet was a simple one and that it would be found in dyes containing similar chemical groups, a number of such compounds were tested. The results were negative. Therefore, an attempt was made to determine the active chemical group by testing 108 dyes, representing each of the different groups of biological dyes (table 1). Because the optimum dilution of crystal-violet was critical, it was expected that the optimum dilution of other dyes having similar differential properties would also be critical. Therefore, enough of each dye was added to the agar base to make dilutions of 1:10,000, 1:100,000, 1:250,000 and 1:500,000. Each dye agar was inoculated heavily with 10 strains of staphylococci which were selected because of their different hemolytic, coagulating and crystal-violet agar reactions. After 36 hours incubation, the colors of the growths were compared with those which had been produced on crystal-violet agar.

Inconclusive results were obtained with most of the dyes, any color changes being associated with *albus-aureus* differences, i.e., related to pigment. However, significant color changes were noted with the following dyes: 5 samples of methyl-violet, 1 of crystal-violet and 1 of Hoffman violet which gave results similar to those obtained with the original sample of crystal-violet; and bromthymol blue which gave color changes parallel to, but chemically different from, those produced on crystal-violet agar. The results with bromthymol blue agars depended upon the indicator property of the dye.

STUDIES OF METHYL-VIOLET AND RELATED VIOLET DYES

These results suggested that the active molecule of the crystal-violet agar reaction is contained in crystal- and methyl-violet and some samples of Hoffman violet. Therefore, tests were made with a larger series of violets. Because of the close relationship between Hoffman and ethyl-violets and, because one dye was

labeled "methyl-violet (methylene violet)," samples from these groups were also included. A total of 60 samples was studied among which were "Dahlia," "Dahlia B," crystal-, methyl-, gentian-, ethyl-, Hoffman and methylene violets. The results are summarized in table 2.

With the exception of a few samples of methyl-violet which, since they were labeled "R," "2R," etc., appeared to be mostly tetramethyl pararosanilin, dyes labeled "methyl," "gentian" and

TABLE 2

Chromatic properties of 60 samples of violet dyes when tested for their value in the crystal-violet agar reaction of staphylococci

MANUFACTURER'S DESIGNATION OF DYES TESTED	ABILITY TO DIFFERENTIATE STAPHYLOCOCCAL GROWTHS				
	Standard		Poor	Slight or none	No parallel
	a*	b†			
Crystal—violet	6		4		
Methyl—violet	24		7		
Gentian—violet	3		1		
Dahlia B	1				
Hoffman violet	1	1		3	
Ethyl—violet		2		3	
Dahlia		1			
Methylene violet					3

* Colored growths on these dye agars were similar to those on standard violet agar. The intensity of the color of an 0.1 per cent solution was approximately equal to that of the standard sample of crystal-violet.

† Colored growths on these dye agars were similar to those on standard violet agar. However, the intensity of the color of an 0.1 per cent solution was equal to only 54 to 75 per cent of the standard sample of crystal-violet. The solutions had rose tints.

"crystal" violet gave color changes typical of crystal-violet. Dyes considered as tetramethyl pararosanilin gave poor color differentiation.

Methylene violets, which included one sample labeled "methyl-violet (methylene violet)," gave atypical color differentiation.

To obtain comparative results with ethylated compounds, such as ethyl or Hoffman violet or Dahlia, it was necessary to use higher concentrations of the dyes. Such media were similar in

intensity of color to those prepared from methylated pararosanilins. With these concentrations, 2 of 5 samples of ethyl-violet, 2 of 5 samples of Hoffman violet and 1 sample labeled "Dahlia" gave color reactions typical of crystal-violet. If many of these dyes are improperly labeled, as suggested by Conn (1929) then the typical color reactions given by some samples may be explained on the basis of their containing crystal-violet or a higher homolog of methyl-violet. The fact that the solutions were rose-colored and that considerably more dye was required to bring the color to match standard crystal-violet agar in intensity indicates that they also contained either ethyl or Hoffman violet (Dahlia) as claimed on the labels. On this assumption, ethylated dyes giving color differentiation typical of crystal-violet are believed to be mixtures containing methylated pararosanilins. If this is true, then the only dyes which give the color reaction are those which contain moderate or large amounts of methylated pararosanilins.

The active chemical group can be still further defined from a consideration of the relationship between methyl-, crystal- and gentian-violets. Methyl-violet is a mixture of tetra-, penta- and hexa-methyl pararosanilin, the exact shade depending upon the proportion of different homologs. The redder shades, designated "R," "2R," etc., which are composed mostly of tetramethyl pararosanilin did not give characteristic color changes. Therefore, the active groups should be sought in the higher homologs. The two higher homologs (pentamethyl and hexamethyl pararosanilin are not clearly differentiated in biological dyes. This is due to the lack of precise control of the degree of methylation, so that samples offered on the open market may contain these two compounds in different proportions and may also contain the tetramethyl compound. On the other hand, crystal-violet which is usually made by a different synthesis is composed almost entirely of the hexamethyl compound. Gentian-violet, a term which is becoming obsolete in this country, is used rather loosely to designate various types of methyl- or crystal-violet.

These observations lend support to the belief that the active group in the crystal-violet agar reaction of staphylococci is either

pentamethyl or hexamethyl pararosanilin, or both. This is further supported by the following observations.

Dyes such as malachite green or auramin, which have only two amino groups, do not react. This shows the importance of the three amino groups. These groups must be substituted, because dyes such as the fuchsins in which they are not, fail to give the color reaction. Ethylated rosanilins and pararosanilins, such as brilliant-green and ethyl-violet, do not give characteristic color changes. However, samples believed to contain either pentamethyl or hexamethyl pararosanilin, or both, give typical color differentiation due to the presence of such compounds. This shows the value of methyl substitution.

Dyes such as Janus green B, methylene-blue, methylene-green, neutral-violet, thymol-blue and bromthymol blue, which contain 4 or more methyl groups, do not react. This proves that it is not methyl substitution alone, but the attachment of methyl groups to another specific group which furnishes the basis for the reaction.

Dyes such as methyl-violet 2R, considered as lower homologs, either do not react or give poor differentiation when they do react. This eliminates tetramethyl pararosanilin. The introduction of further methyl or other groups into crystal-violet as in methyl-green, iodine-green and methyl-blue, prevents the color reaction. This demonstrates the unfavorable effect of further changes in the crystal-violet molecule.

Because it is more likely to be uniform, it is suggested that Commission Certified crystal-violet be used for the preparation of crystal-violet agar.

SUMMARY

Heavy inoculation is necessary to secure growth of staphylococci on crystal-violet agar.

Solutions of crystal-violet used for the preparation of crystal-violet agar should be freshly prepared. The optimum dilution is between 1:300,000 and 1:350,000.

Although representatives of the different groups of biological dyes were tested with staphylococci, color changes similar to those

produced on crystal-violet media were obtained only with media prepared from dyes containing methyl-, gentian- or crystal-violet.

The active group of the dye used in the crystal-violet agar reaction of staphylococci was found to be either pentamethyl or hexamethyl pararosanilin, or both.

CONCLUSIONS

The violet agar reaction of staphylococci is highly specific as far as the dye is concerned and depends upon a reaction of growth products of staphylococci with either pentamethyl pararosanilin or hexamethyl pararosanilin. To insure best results, only fresh solutions of crystal-violet, preferably Commission Certified, should be used for the preparation of crystal-violet agar.

REFERENCES

CHAPMAN, G. H., AND BERENS, C. 1935 Jour. Bact., **29**, 437.
CONN, H. J. 1929 Biological Stains, Geneva, New York, pp. 100–1.

STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

VIII. UTILIZATION OF GLUTAMIC ACID BY THE DIPHTHERIA BACILLUS

J. HOWARD MUELLER

Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Massachusetts

Received for publication April 7, 1936

In earlier papers (1935, a, b and c) the writer has shown that mixtures of certain amino acids will replace adequately the peptone fraction of a meat-infusion peptone broth for the propagation of two strains of diphtheria bacillus which were investigated. While there were differences in the requirements of the two, they were alike in their use of glutamic acid, and, to produce maximal growth, quantities of this amino acid greatly in excess of those of any of the other essential amino acids had to be added to the medium. This fact suggested the possibility that perhaps some impurity, rather than glutamic acid itself, might prove to be responsible for the effect. In view of the fact that the quantity of growth increased along an almost straight line up to a concentration of 0.5 per cent glutamic acid hydrochloride, and that further additions produced still greater increases, although not with the same direct proportionality, while of such other essential amino acids as tryptophane, cystine and methionine quantities of the order of 0.01 per cent were adequate for maximal growth, it was clear that 1 to 2 per cent of an active impurity in the glutamic acid might account completely for its apparent action. Moreover, although repeated recrystallizations failed to change the activity of the compound, the difficulty of obtaining many of the amino acids from natural sources in a completely pure form still left the possibility of the presence of an impurity to be excluded by other means.

STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

VIII. UTILIZATION OF GLUTAMIC ACID BY THE DIPHTHERIA BACILLUS

J. HOWARD MUELLER

Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Massachusetts

Received for publication April 7, 1936

In earlier papers (1935, a, b and c) the writer has shown that mixtures of certain amino acids will replace adequately the peptone fraction of a meat-infusion peptone broth for the propagation of two strains of diphtheria bacillus which were investigated. While there were differences in the requirements of the two, they were alike in their use of glutamic acid, and, to produce maximal growth, quantities of this amino acid greatly in excess of those of any of the other essential amino acids had to be added to the medium. This fact suggested the possibility that perhaps some impurity, rather than glutamic acid itself, might prove to be responsible for the effect. In view of the fact that the quantity of growth increased along an almost straight line up to a concentration of 0.5 per cent glutamic acid hydrochloride, and that further additions produced still greater increases, although not with the same direct proportionality, while of such other essential amino acids as tryptophane, cystine and methionine quantities of the order of 0.01 per cent were adequate for maximal growth, it was clear that 1 to 2 per cent of an active impurity in the glutamic acid might account completely for its apparent action. Moreover, although repeated recrystallizations failed to change the activity of the compound, the difficulty of obtaining many of the amino acids from natural sources in a completely pure form still left the possibility of the presence of an impurity to be excluded by other means.

Clearly the most direct evidence could be secured by the substitution of synthetic glutamic acid for the natural compound. At the time of our earlier publication this substance was not available, and the question was left open. Recently, through the courtesy of Prof. Carl S. Marvel of the Chemical Department of the University of Illinois, a small quantity of this material[1] was obtained, and it has been possible to show quite definitely

TABLE 1

	H. Y. STRAIN		PARK 8 STRAIN*
l-Cystine	1.0 mgm.	l-Cystine	2.0 mgm.
l-Tryptophane	1.0 mgm.	dl-Methionine	2.0 mgm.
dl-Methionine	2.0 mgm.	Glycine	5.0 mgm.
l-Histidine-HCl	1.0 mgm.	dl-Valine	10.0 mgm.
Glycine	5.0 mgm.	dl-Leucine	5.0 mgm.
dl-Valine	10.0 mgm.	Glycerol	0.05 cc.
dl-Leucine	5.0 mgm.	NaCl	50.0 mgm.
Ethyl alcohol	0.05 cc.	$Na_2HPO_4 \cdot 2H_2O$†	25.0 mgm.
NaCl	50.0 mgm.	KH_2PO_4	3.5 mgm.
$Na_2HPO_4 \cdot 2H_2O$	25.0 mgm.†	$MgCl_2 \cdot 6H_2O$	3.0 mgm.
KH_2PO_4	3.5 mgm.	Liver elute‡	0.25 cc.
$MgCl_2 \cdot 6H_2O$	3.0 mgm.		
Liver elute	0.25 cc.‡		

* A strain obtained from the Massachusetts Antitoxin Laboratory, and sent them by the National Institute of Health in Washington. There are very considerable differences in growth requirement shown by strains of the Park 8 organism from various laboratories, which will be the subject of a report in the near future.

† The sodium phosphate is now added because of its buffer effect, and obviously in its presence KCl could be substituted for the KH_2PO_4 which was previously included to supply both the K and PO_4 requirements.

‡ Prepared as previously described (Mueller, 1935 c).

that the effect is due actually to *d*-glutamic acid, since the *dl* compound shows almost exactly half the activity of the natural form.

[1] The writer is greatly indebted to Prof. Marvel for his courtesy in supplying this material, the synthesis of which offers no little difficulty. The specimen was stated to melt at 191° (uncorrected) and to contain 19.35 per cent Cl against the calculated value of 19.36 per cent.

EXPERIMENTAL

The two strains of the diphtheria organism previously studied were used for the test. Control media were prepared as shown

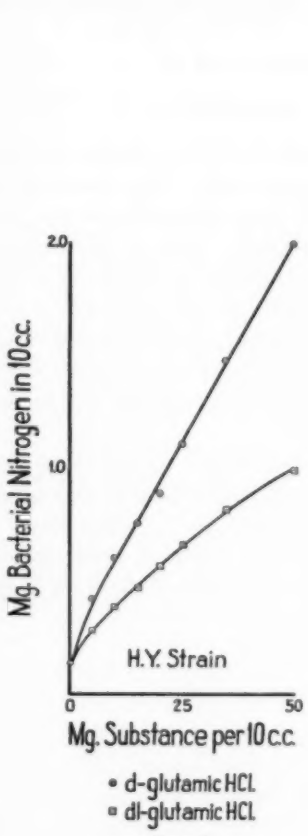


FIG. I

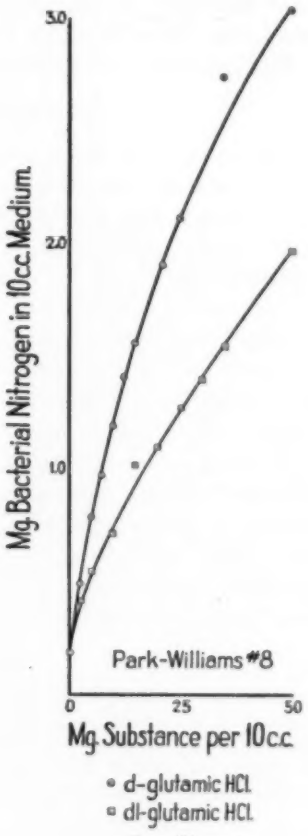


FIG. II

in table 1, following the requirements already defined for these strains. The quantities given are for 10 cc. medium.

Varying amounts of *d* and of *dl* glutamic acid hydrochloride were then added to a series of tubes each containing 10 cc. of the

respective control media, and the reactions adjusted to pH 7.6 to 7.8. After sterilization in the autoclave, the tubes were inoculated, incubated in a slanting position at 35° for 60 to 64 hours, and the relative amounts of growth then estimated by the total nitrogens of the centrifuged and washed bacteria in the usual way (Mueller, 1935 a).

The results are summarized in figures I and II.

DISCUSSION AND SUMMARY

The curves with the natural *d*-glutamic acid are similar for both strains to those obtained in our earlier work. The synthetic or *dl* compound has, weight for weight, approximately 50 per cent of the effect produced by the *d* material. This is shown most strikingly by the H. Y. strain. In the case of the Park 8, the average effect is somewhat greater than 50 per cent, but not to an extent that would indicate any considerable utilization of the *l* isomer. It is probable that a repetition of the experiment would have brought the points on a somewhat better line, but the general trend of the results is perfectly clear.

These observations would appear to supply direct proof that glutamic acid can serve as the principal source of nitrogen in the growth of the diphtheria bacillus, and confirm the conclusions of Abt (1925), reached through an entirely different experimental procedure, that it does so serve as a component of peptone in the usual laboratory media.

REFERENCES

ABT., G. 1925 Ann. de l'Inst. Pasteur, **39**, 387.
MUELLER, J. H. 1935 a Jour. Bact., **29**, 383.
MUELLER, J. H. 1935 b Jour. Bact., **29**, 515.
MUELLER, J. H. 1935 c Jour. Bact., **30**, 513.
MUELLER, J. H., AND KAPNICK, I. 1935 Jour. Bact., **30**, 525.

THE INDOL TOLERANCE OF CERTAIN STRAINS OF THE COLON-AEROGENES GROUP[1]

RALPH L. FRANCE

Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

Received for publication March 11, 1936

INTRODUCTION

Gordon and McLeod (1926) reported that small concentrations of indol inhibited the growth of certain bacteria. They suggested that: "Indol production may well be the mechanism by which *Bact. coli* maintains its dominant position in the fecal flora." Kilborn, Pierce and Tittsler (1929) reported retardation of bacterial reproduction by indol, and in some species of enteric bacilli an actual killing. They reported that *Escherichia coli* was the most resistant of the organisms that were studied. These results suggested to the writer that a study of the indol tolerance of the colon-aerogenes organisms might supply data of practical value. It was thought that *Escherichia coli*, as a normal inhabitant of the intestines, might have a greater tolerance to indol than the non-fecal members of the group. Preliminary reports of this study were published in 1934 and 1935.

Since this investigation was begun, Tittsler and Sandholzer (1935) have reported the results of a study of the bacteriostatic action of indol on Gram-negative enteric bacilli, and on certain cocci. They found that indol in a dilution of 1:1500 inhibited all the strains of coli-aerogenes organisms used. They stated that: "In general, members of the *Escherichia* and *Aerobacter* genera, and the *Escherichia-Aerobacter* 'intermediate' group, were inhibited by approximately the same concentration of indol."

The present study is a report of the indol tolerance of 173 strains

[1] Published as Contribution No. 246, Massachusetts Agricultural Experiment Station.

of fecal type *Escherichia coli*, 155 strains of *Aerobacter aerogenes*, and 83 strains of "intermediates."

MEDIA AND METHODS

The cultures used were from various sources. Of the fecal *Escherichia coli* strains, 119 were from feces and 54 were from water. All of the 155 *Aerobacter aerogenes* strains were isolated from water. Of the "intermediate" strains, 70 were obtained from water and 13 from feces. These organisms were placed in the three groups on the basis of their reactions to the Voges-Proskauer, methyl red, sodium citrate, and uric acid tests. These reactions were found to be constant on repeated tests.

Solutions of 1:10 and 1:20 of indol (Will Corporation) in 70 per cent alcohol were prepared. These solutions were self-sterilizing in 48 hours in the ice box. Standard lactose broth was distributed in flasks in 100 cc. portions. After sterilization the flasks were incubated at 37°C. to assure sterility. Rubber stoppers were used in place of cotton stoppers to prevent volatilization of the indol during subsequent incubation. Before inoculation an amount of broth was removed, by means of sterile serological pipettes, equivalent to the amount of indol solution added to give the desired dilution. The flasks were then inoculated by adding one drop of a suspension of the organisms of a density equivalent to 1.5 of the McFarland nephelometer scale. Sufficient amounts of the 1:10 and 1:20 solutions of indol were then added to make dilutions of 1:1500, 1:1800, 1:2000, 1:2300, 1:2500, and 1:3000. Each culture was inoculated in duplicate flasks in each dilution. Readings were made after 24 and 48 hours of incubation at 37°C. All of the strains were tested at least twice, and 40 per cent of them were tested three times.

RESULTS

Table 1 gives a summary of the results of the tests on the 411 cultures. It will be observed that all of the cultures grew in a 1:3000 dilution while all were inhibited by dilution of 1:1500 of the indol. Ninety per cent of the cultures were inhibited by dilutions ranging from 1:1800, to 1:2300 inclusive.

Table 2 gives a summary of the results obtained with each of the groups of organisms. It will be observed that the range of tolerance within each group overlaps the range of tolerance of each of the other groups, so that there is no marked variation of tolerance between the strains of various groups.

TABLE 1

Inhibition of growth of colon-aerogenes bacteria by various dilutions of indol

CONCENTRATION OF INDOL	CULTURES INHIBITED	
	Number	Per cent
1:1500	411	100
1:1800	401	98
1:2000	351	85
1:2300	202	49
1:2500	29	7
1:3000	0	0

TABLE 2

Indol tolerance of the colon-aerogenes groups

GROUP	DILUTION OF INDOL AND NUMBER OF STRAINS INHIBITED						TOTAL STRAINS TESTED
	1:1500	1:1800	1:2000	1:2300	1:2500	1:3000	
E. coli	173	169	151	69	0	0	173
A. aerogenes	155	155	135	92	25	0	155
"Intermediates"	83	77	65	41	4	0	83

DISCUSSION

The object of this experiment, as stated in the introduction, was to determine whether there existed a sufficiently wide variation in the indol tolerance of the various members of the coli-aerogenes group to be of practical value. The results indicate that these groups are so similar in their tolerance for indol, that no practical application can be made.

In the investigations preliminary to this study an attempt was made to add the various amounts of the indol solutions to flasks of melted Endo's agar. Plates were then poured from these flasks and rapidly cooled. Suspensions of the organisms were

streaked on the plates, and incubation was conducted at 37°C. for 48 hours. The results obtained showed inhibition at dilutions somewhat higher than that observed with the lactose broth. However, repetitions of the tests failed to give consistent results. This was probably due to the fact that it is impossible to get an equal distribution of the indol throughout the agar.

An interesting observation was made throughout the entire experiment. When the indol solution was added to the flasks there was a noticeable crystallization of the indol in the solution. This happened in every instance. In an endeavor to make certain that it was the indol which was crystallizing, the writer slowly added water to the alcoholic solutions of indol. It was found that as soon as the alcohol concentration became less than 35 per cent the indol would precipitate out. If this same phenomenon occurred in the lactose broth the writer is somewhat puzzled as to just what caused the inhibition. There is the possibility that sufficient indol remained in solution to cause the inhibition of growth of the organisms. Or, again, it might be possible that the volatilization of the indol was the factor causing inhibition.

In support of this supposition, Tittsler and Sandholzer (1935) cited the work of Mitolo who found that the vapor from 5 to 10 mgm. of indol prevented the growth of bacteria seeded on the surface of agar.

REFERENCES

FRANCE, RALPH L. 1934 Massachusetts Agricultural Experiment Station, Bull. No. 305, p. 16.

FRANCE, RALPH L. 1935 Massachusetts Agricultural Experiment Station, Bull. No. 315, p. 22.

GORDON, J., AND MCLEOD, J. W. 1926 Jour. Path. and Bact., **29**, 13–27.

KILBORN, PIERCE, AND TITTSLER: Cited from Tittsler and Sandholzer, 1935.

TITTSLER, RALPH P., AND SANDHOLZER, LESLIE, A. 1935 Jour. Inf. Dis., **57**, 64–69.

THE PHENOL COEFFICIENT AS A MEASURE OF THE PRACTICAL VALUE OF DISINFECTANTS[1]

J. C. VARLEY AND G. F. REDDISH

St. Louis College of Pharmacy, St. Louis, Missouri[2]

Received for publication April 13, 1936

The phenol coefficient test has been employed for determining the germicidal efficiency of disinfectants for over thirty years, since Rideal and Walker developed their original method in 1903. The Hygienic Laboratory Method (1921) is a modification of the Rideal-Walker Method, and the Food and Drug Administration test (1931) is a combination of the best features of both. The phenol coefficient technique as employed today does not differ fundamentally from the original test as described by Rideal and Walker, and, in one form or another, it is being used as a means of testing disinfectants in every civilized country in the world.

For approximately twenty years the United States Department of Agriculture has used the phenol coefficient test in the regulation of disinfectants in this country, and it is still the most satisfactory method available for this purpose. In addition to being a satisfactory index of the germicidal value of phenol-like disinfectants, the phenol coefficient is used as a basis for determining the dilutions which may safely be employed in practice. This dilution is calculated by multiplying the phenol coefficient by twenty, which will give a germicidal efficiency equal to 5 per cent carbolic acid. Since, in this country, 5 per cent carbolic acid is accepted as the standard of excellence for general disinfection, phenol-like compounds must be recommended for general use in dilutions equal to 5 per cent phenol in germicidal efficiency. This method of

[1] Read before the twenty-second annual meeting of the National Association of Insecticide and Disinfectant Manufacturers, New York, December 10, 1935.

[2] The laboratory work in connection with this investigation was conducted in the Bacteriology Laboratory of Baird and McGuire, Inc., St. Louis, Mo.

calculating dilutions for general use for this class of disinfectants—twenty times the phenol coefficient—is employed by the United States Food and Drug Administration in the regulation of germicides for use on inanimate objects and it is found to be quite satisfactory.

The use of this factor—twenty times the phenol coefficient—affords a wide margin of safety. As pointed out by Reddish (1935), the dilutions calculated on this basis are of sufficient strength to kill all pathogenic microörganisms which cause epidemics. Most bacteria which cause epidemic diseases are similar in resistance to *Eberthella typhosa*, the test organism in the phenol coefficient test. The dilution of the disinfectant which kills *Eberthella typhosa* is usually sufficiently germicidal to kill most of the disease-producing microörganisms. There are some, however, which are more resistant and it is these which are considered in calculating the dilutions to be recommended for use in practice.

Klarmann (1934) has indicated that this factor is not enough, so that some disinfectants when diluted to twenty times their phenol coefficient will not kill certain important disease-producing bacteria, especially *Streptococcus hemolyticus*. Reddish (1935), however, has listed other factors of safety which must be noted in this connection, and he considers these more than enough to supply a satisfactory margin of safety.

The writers have devoted themselves to a practical study of this problem. We have attempted to answer the question: "Will a phenol-like disinfectant, in a dilution equal to twenty times its phenol coefficient, be effective in killing pathogenic microörganisms under practical conditions of use?" Another question we have attempted to answer in this investigation is whether high-phenol-coefficient disinfectants and low-coefficient disinfectants will be equally effective when diluted to twenty times their respective phenol coefficients. For this purpose we have used representatives of two classes of phenol-like disinfectants, namely, coal tar and cresylic acid, or, as they are commonly known, "Tar Oil" and Cresol Compound Disinfectants. The first represents the emulsifiable type and forms a milky suspension in water, while the other forms clear solutions with water.

The phenol coefficient of each disinfectant was first determined by the Food and Drug Administration Method (1931). The figures obtained were used as a basis for calculating the dilution for general use—twenty times the phenol coefficient—and these dilutions were made with distilled water. The coal tar disinfectants employed had phenol coefficients ranging from 2 to 20 (coefficients 2, 3, 4, 5, 6, 7, 8, 10, and 20), while the cresol compound disinfectants used ranged in strength from coefficients 2 to 5. *Eberthella typhosa* was the only test organism employed in obtaining these phenol coefficient figures. No attempt was made in any case to determine the *Staphylococcus aureus* phenol coefficient, since the above calculations for practical dilutions are always based on the *Eberthella typhosa* figure.

Since there is no standard method for testing disinfectants under practical conditions, it was necessary to develop an entirely new test and establish certain arbitrary conditions for the purpose of this special investigation. The following procedure was employed in this study:

An area of 100 square inches was marked off on special flooring material obtained for the purpose. This was washed thoroughly, rinsed, and allowed to dry. Broth cultures of the test organisms employed were diluted so that 1 cc. contained approximately 100,000 organisms. One cubic centimeter of the culture suspension was spread over the entire 100 square inch test area by means of a small rubber spreader (which is 2 inches wide). Five minutes later a cotton swab, previously moistened in sterile water, was rubbed back and forth over representative portions of the test area twelve times, after which it was twirled vigorously in 15 cc. of melted agar, cooled to 45°C., the material and agar thoroughly mixed by rotating and poured into a sterile Petri dish. This was the control plate; it was made at the beginning of each test. Immediately after the control swabbing, 3 cc. of the diluted disinfectant were applied to the test area and spread over the entire 100 squre inches by means of the rubber spreader. Smears were taken with a moistened cotton swab at the end of 5, 10, 15, 20, 25, and 30 minutes (using the same procedure employed in taking the control culture), inoculated into 15 cc. of melted agar and poured into Petri dishes. All plates were then incubated at 37°C. for 48 hours. At the end of this time the plates were observed for growth of the test organ-

isms, and colonies counted when present. Each plate, with the exception of the control plate, was then streaked with a fresh 24-hour broth culture of the test organism and incubated for 24 hours, to test for bacteriostatic action in the agar. The test was conducted at ordinary room temperature.

Three test organisms were employed in these tests, namely, *Staphylococcus aureus, Escherichia coli* and *Eberthella typhosa. Staphylococcus aureus* is representative of the most resistant of the non-sporing organisms, including *Streptococcus hemolyticus* and *Streptococcus viridans*, while *Escherichia coli* and *Eberthella typhosa* represent pathogenic microörganisms of average resistance. Much larger numbers of these organisms were added to the test area than are found ordinarily under practical conditions, because we sought to establish maximum or even exaggerated conditions. By the use of these three test organisms, representing the most resistant microörganisms (excluding spores) as well as those of average resistance, it is possible to draw reasonable conclusions as to the value of disinfectants for killing those bacteria which cause epidemic diseases, under practical conditions.

The flooring materials used were representative of those ordinarily met with in practice. Wood, linoleum, asphalt, rubber, and enameled floorings were employed. These floorings fall into two classifications, porous and non-porous, the asphalt and wood being porous, and the rubber, linoleum, and enameled floors, non-porous. For physical reasons, the non-porous materials were used in the majority of these tests because more accurate results were obtained with them than with the others. The type of floor, however, had no bearing on the results of the test, in so far as germicidal activity of the disinfectant was concerned, since this activity was exerted in the porous floors just as readily as on the non-porous materials.

Another factor in this test which must be considered is the dilution which takes place when the disinfectant solution is added to the test area following the application of the 1 cc. of culture suspension. Since 3 cc. of disinfectant were added five minutes after 1 cc. of culture suspended in water was applied, and before it had dried, there is an immediate further dilution of the dis-

infectant of approximately 30 per cent or somewhat less. In some of the tests the original disinfectant dilution was made more concentrated to take into account this further dilution, but in most of them this factor was ignored, although the additional dilution worked to the disadvantage of the disinfectant. The reason for this was that even in spite of this further dilution with the culture suspension on the test area, the disinfectant was accomplishing the desired effect. This served to demonstrate again the very wide margin of safety that is allowed in making the dilution of the disinfectant originally. This adjustment of the dilution to take into account the addition of water was resorted to in only fourteen out of almost a hundred tests.

In interpreting the data presented here, it must be remembered that in every test very large numbers of the test organisms were employed, far more than are met with ordinarily in practice. In practically every case the control count—that is, the number of organisms removed from the flooring before the disinfectant was applied—was "uncountable." The killing of these very large numbers of representative disease-producing organisms within the time periods indicated illustrates the high germicidal efficiency of the dilutions of the disinfectants employed.

EXPERIMENTAL

Coal tar group

Test organism: Staphylococcus aureus. The tests conducted on the Coal Tar Group of disinfectants, which ranged in strength from phenol coefficients 2 to 20, gave the results given in table 1 when tested against a standard resistant strain of *Staphylococcus aureus.*

In 65 per cent of the tests this organism was killed within 5 minutes. In 13 per cent of the tests complete killing resulted within less than 10 minutes, but not in 5 minutes; in 18 per cent of the tests complete kill resulted within 20 minutes, but not in 10 minutes; while in 4 per cent of the tests all were killed within 30 minutes.[3] Under the conditions of this test the average

[3] In this one test results were obtained for the 30-minute time period only.

length of time necessary to kill these exaggerated numbers of this resistant organism was approximately 9 minutes.

Test organism: Escherichia coli. When *Escherichia coli* was employed as the test organism in experiments with coal tar dis-

TABLE 1

Test organism, Staphylococcus aureus. Disinfectant, coal tar

COEFFICIENT	DILUTION	TEMPERATURE	CONTROL PLATE COUNT	PLATE COUNT AFTER MINUTES						TYPE OF FLOOR	REMARKS
				5	10	15	20	25	30		
		°C.									
2	1:40	23	Uncountable	0	0	0	0	0	0	Enamel	
3	1:60	23	Uncountable	23	12	6	0	0	0	Rubber	
3	1:60	24	4,000	0	0	0	0	0	0	Enamel	
3	1:60	23	Uncountable	35	20	11	0	0	0	Rubber	
3	1:60	26	Uncountable	0	0	0	0	0	0	Rubber	
4	1:60	23	Uncountable	0	0	0	0	0	0	Enamel	D.A.*
5	1:75	23	Uncountable	0	0	0	0	0	0	Rubber	D.A.*
5	1:75	23	Uncountable	0	0	0	0	0	0	Varnished wood	D.A.*
5	1:100	26	Uncountable	4	0	0	0	0	0	Rubber	
5	1:100	23	Uncountable	0	0	0	0	0	0	Rubber	
5	1:75	26	Uncountable		0	0	0	0	0	Enamel	D.A.*
6	1:120	23	Uncountable	240	37	18	0	0	0	Rubber	
6	1:90	23	Uncountable	0	0	0	0	0	0	Rubber	D.A.*
6	1:120	26	Uncountable	0	0	0	0	0	0	Rubber	
6	1:120	23	Uncountable						0	Linoleum	
7	1:140	23	Uncountable	0	0	0	0	0	0	Rubber	
7	1:140	25	Uncountable	0	0	0	0	0	0	Rubber	
7	1:140	26	Uncountable	0	0	0	0	0	0	Rubber	
10	1:150	23	Uncountable	0	0	0	0	0	0	Rubber	D.A.*
20	1:300	23	Uncountable	0	0	0	0	0	0	Enamel	D.A.*
20	1:400	23	3,500	0	0	0	0	0	0	Rubber	
20	1:400	26	Uncountable	40	15	4	0	0	0	Rubber	
20	1:400	20	Uncountable	50	0	0	0	0	0	Rubber	

* D.A. Dilution of the disinfectant was adjusted to take into account the further dilution with the culture suspension.

infectants (the same that were used in the tests with *Staphylococcus aureus*) the results shown in table 2 were obtained.

In 50 per cent of the tests complete killing resulted in (or within) 5 minutes; 22 per cent showed complete killing within 10 minutes, but not in 5 minutes; 22 per cent killed within 20 min-

utes, but not in 10 minutes; while 7 per cent killed within 30 minutes, but not in 20 minutes (with one exception). Under the

TABLE 2

Test organism, Escherichia coli. Disinfectant, coal tar

COEFFICIENT	DILUTION	TEMPERATURE	CONTROL PLATE COUNT	PLATE COUNT AFTER MINUTES						TYPE OF FLOOR	REMARKS
				5	10	15	20	25	30		
		°C.									
2	1:40	21	400	0	0	0	0	0	0	Rubber	
2	1:40	21	1,992	0	0	0	0	0	0	Rubber	
2	1:40	26	Uncountable	0	0	0	0	0	0	Rubber	
3	1:45	23	Uncountable	0	0	0	0	0	0	Enamel	D.A.*
3	1:45	23	Uncountable	0	0	0	0	0	0	Rubber	D.A.*
3	1:60	23	Uncountable	23	0	0	0	0	0	Varnished wood	
3	1:60	23	Uncountable	34	0	0	0	0	0	Rubber	
5	1:100	20	Uncountable	15	0	0	0	0	0	Varnished wood	
5	1:100	23	Uncountable	15	0	0	0	0	0	Varnished wood	
5	1:100	20	Uncountable	0	0	0	0	0	0	Rubber	
5	1:100	23	Uncountable	48	73	49	0	0	0	Varnished wood	
5	1:100	24	Uncountable	2	7	0	0	0	0	Tin	
6	1:120	24	Uncountable	25	0	0	0	0	0	Rubber	
6	1:120	24	Uncountable	1	0	0	0	0	0	Rubber	
7	1:105	23	Uncountable	0	0	0	0	0	0	Enamel	D.A.*
8	1:160	24	Uncountable	560	480	140	100	0	0	Rubber	
8	1:160	25	Uncountable	0	64(?)	0	0	0	0	Glass	
8	1:160	25	Uncountable	280	230	200	47		32	Rubber	
8	1:160	24	Uncountable	0	0	0	0	0	0	Tin	
10	1:150	23	Uncountable	0	0	0	0	0	0	Enamel	D.A.*
10	1:200	24	Uncountable	0	0	0	0	0	0	Tin	
20	1:400	23	Uncountable	0	0	0	0	0	0	Glass	
20	1:400	23	Uncountable	0	2(?)	0	0	0	0	Rubber	
20	1:400	23	270	0	1(?)	0	0	0	0	Tin	
20	1:400	23	190	0	0	0	0	0	0	Glass	
20	1:300	25	Uncountable	0	0	0	0	0	0	Glass	D.A.*
20	1:300	25	Uncountable	300	100	30	0	0	0	Rubber	D.A.*

conditions of this test, *Escherichia coli* in exaggerated numbers was completely killed in an average time of approximately 9 minutes.

Test organism: Eberthella typhosa. When *Eberthella typhosa* was used as the test organism in tests on the same coal tar disinfectants in dilutions 20 times the phenol coefficient, all of the organisms were killed within 5 minutes in every case.

Cresol compound group

Test organism: Staphylococcus aureus. The tests conducted on cresol compound disinfectants with phenol coefficients ranging

TABLE 3

Test organism, Staphylococcus aureus. Disinfectant, cresol and cresylic acid

COEFFICIENT	DILUTION	TEMPERATURE	CONTROL PLATE COUNT	PLATE COUNT AFTER MINUTES						TYPE OF FLOOR	REMARKS
				5	10	15	20	25	30		
		°C.									
2	1:40	20	Uncountable	0	0	0	0	0	0	Rubber	
5	1:100	23	Uncountable	0	0	0	0	0	0	Rubber	
5	1:100	23	Uncountable	0	0	0	0	0	0	Rubber	
5	1:100	20	1,936	52	0	0	0	0	0	Linoleum	
5	1:200*	20	4,000	0	0	0	0	0	0	Rubber	D.A.*

* In this test the dilution used was twice that ordinarily employed.

TABLE 4

Test organism, Escherichia coli. Disinfectant, cresol and cresylic acid

COEFFICIENT	DILUTION	TEMPERATURE	CONTROL PLATE COUNT	PLATE COUNT AFTER MINUTES						TYPE OF FLOOR
				5	10	15	20	25	30	
		°C.								
2	1:40	20	Uncountable	1	0	0	0	0	0	Rubber
2	1:40	23	Uncountable	0	0	0	0	0	0	Tin
5	1:100	20	Uncountable	0	0	0	0	0	0	Rubber
5	1:100	23	Uncountable	0	0	0	0	0	0	Glass
5	1:100	20	516	3	0	0	0	0	0	Asphalt
5	1:100	23	Uncountable	0	0	0	0	0	0	Rubber

from 2 to 5 gave the results shown in table 3 when tested against *Staphylococcus aureus.*

In 80 per cent of the tests this organism was killed within 5 minutes, while in 20 per cent of the tests 10 minutes were required to effect complete killing of all the organisms.

Test Organism: Escherichia coli. The tests conducted on cresol compound disinfectants with phenol coefficients ranging from 2 to 5 gave the results shown in table 4 when tested against *Escherichia coli.*

In 67 per cent of the tests *Escherichia coli* was killed within 5 minutes, while in 33 per cent of the tests complete killing of all the organisms resulted within 10 minutes, but not in 5 minutes.

Test Organism: Eberthella typhosa. Under the conditions of the test, *Eberthella typhosa* was killed within 5 minutes in every test in dilutions 20 times the phenol coefficient.

5 per cent carbolic acid

Five per cent phenol was used on two kinds of floors with *Escherichia coli* as the test organism. Only two tests were made. In one test all of the organisms were killed within 5 minutes, while in the other all but three were killed in 5 minutes, and all within 10 minutes.

In connection with these tests it must be noted that the numbers of organisms surviving after the various time periods were very small. In comparison with the very large numbers applied to the floor surfaces, as indicated in the control counts, little significance can be attached to the few that were still living after these time periods. If numbers of organisms had been applied to the test surfaces comparable to the number found in practice, it is almost certain that *all* of the test organisms would have been killed within the shortest time period of the test, 5 minutes. Since it is difficult, if not impossible, to simulate accurately the numbers of microörganisms found under practical conditions, the expediency of adding exaggerated numbers is justified. However, in evaluating the results obtained in these tests this factor must be considered. When this is done it is evident that the disinfectants employed in these tests in the dilutions used completely kill as many or more bacteria as are found under practical conditions.

In order to prove this, several tests were made to determine the number of bacteria found on ordinary dirty floors, with the results shown in table 5.

The cultures were taken from these dirty floors in the manner described above. Six such tests gave an average of 1700 bacteria on that part of 100 square inches covered by the method of taking the smears (or cultures) as previously described. In some of the tests reported above the control count approximated the count obtained from ordinary floors, and in these instances there was complete killing of the test organisms within 5 minutes in almost every case. Of eleven such tests, only two did not kill all of the test organisms within 5 minutes, and in these two cases the counts after 5 minutes, were quite low, 3 and 52 respectively. In one case the control count was 4000, and all of the test organ-

TABLE 5

Counts made from various floors in actual use

Dirty floors

STAPHYLOCOCCUS AUREUS	TOTAL NUMBER OF ORGANISMS
200	2,000
150	2,000
100	1,500
30	1,000
75	2,000
50	1,500
Total.....605	10,000
Average....100	1,700

isms were killed within 5 minutes, while in another the control count was 1936, and 52 organisms remained after 5 minutes. Such irregularity is to be expected, but on the whole it is proved that, when the control counts approximate the counts obtained in practice from ordinary floors, all of the organisms are killed within approximately 5 minutes or less.

SUMMARY AND CONCLUSIONS

The results presented here show that, when coal tar disinfectants and cresylic acid disinfectants are diluted to 20 times their phenol coefficient, they are effective in killing representative pathogenic microörganisms under practical conditions of use.

It is further shown that disinfectants of varying strengths, that is, whether they have a high or low phenol coefficient, have the same germicidal activity and are equally effective under practical conditions, when diluted to twenty times their respective phenol coefficients.

As a result of these tests it is concluded that:

(1) The phenol coefficient figure is a suitable measure of the practical value of phenol-like disinfectants when the factor "twenty times the phenol coefficient" is employed for calculating the dilutions for use in practice.

(2) Disinfectant solutions made up to twenty times their phenol coefficient are of sufficient strength to kill very large numbers of disease-producing microörganisms under practical conditions.

(3) When dilutions for use are made on this basis, coal tar disinfectants and cresylic disinfectants should be equally germicidal.

(4) When phenol-like disinfectants are diluted in this way they should be as effective in killing disease-producing microorganisms under practical conditions as is 5 per cent carbolic acid.

REFERENCES

Hygienic Laboratory Bulletin, No. 82, April, 1912; Disinfectant Testing by the Hygienic Laboratory Method, Reprint 675, Public Health Reports, **36**, No. 27, July 8, 1921.

KLARMANN, E. 1934 Phenol coefficient—What value? Soap, **10**, 93, October.

REDDISH, GEORGE F. 1935 The significance of the phenol coefficient. Soap, **11**, 95, March.

RIDEAL, S., AND WALKER, J. T. A. 1903 Examination of disinfectants. Jour. San. Inst., **24**, 424–441.

RIDEAL, S., AND WALKER, J. T. A. 1921 Approved technique of the Rideal-Walker test. London.

United States Food and Drug Administration Methods for Testing Antiseptics and Disinfectants. Circular No. 198, December, 1931.

THE IDENTIFICATION OF THE HEMAGGLUTININ OF THE JACK BEAN WITH CONCANAVALIN A

JAMES B. SUMNER AND STACEY F. HOWELL

Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca, New York

Received for publication March 15, 1936

INTRODUCTION

In December, 1935, we identified the hemagglutinin of the jack bean (Assmann, 1911) with the crystallizable globulin concanavalin A (Sumner and Howell, 1935; Sumner, Howell and Zeissig, 1935). In spite of a large amount of research carried out since the discovery of the first hemagglutinin by Dixson in 1887 this is the first agglutinin to be isolated in crystalline form. The jack bean hemagglutinin is a protein of unique properties, since it precipitates glycogen and mucoproteins and agglutinates not only the erythrocytes of certain animal species, but also fat emulsions, starch granules, yeast cells and certain bacteria.

THE PREPARATION OF CONCANAVALIN A

Crystalline concanavalin A is easily prepared by the method described below. When dissolved in saturated sodium chloride it is able to withstand room temperature for several months without any considerable loss in potency. The solution can be evaporated to dryness by air blast at 30°C. without suffering very much denaturation.

Stir 100 grams of finely powdered jack bean meal with 500 cc. of 32-per-cent acetone and filter in an ice chest for 4 or 5 hours, or overnight. Place the residue in a beaker, stir with 500 cc. of 30-per-cent alcohol and filter at room temperature. Now extract the concanavalin A from the residue with 400 cc. of a solution containing 1 per cent sodium chloride and 0.1 per cent neutral phosphate and filter. Extract the residue with 250 cc.

of 5-per-cent sodium chloride and filter. Combine the two last filtrates, add toluene and dialyze against distilled water in three collodion bags for 24 hours. Centrifuge off the crystals of concanavalin A and wash once with 10-per-cent sodium chloride solution. Dissolve the crystals in a small amount of 0.1 N hydrochloric acid. After standing for about one-half hour add a small quantity of neutral phosphate solution and then exactly enough 0.1 N sodium hydroxide to neutralize the hydrochloric acid previously added. Dialyze the material for several hours and centrifuge off the crystals of concanavalin A.

The material is purified by dissolving in a small amount of saturated sodium chloride at 40°C. It is usually necessary to add some solid sodium chloride and to stir. This solution is filtered by gravity. The yield at this point, on a dry weight basis, is 2.5 grams. If it is desired, the concanavalin A can be separated out by dialyzing the solution against distilled water and the material dissolved in concentrated, warm sucrose or warm 70-per-cent glycerol and later separated by dialysis. None of these procedures gives a product entirely free from carbohydrate.

We have found that about 3 grams of concanavalin A can be extracted from 100 grams of finely powdered jack bean meal by the use of 0.1 N hydrochloric acid, hence the purification possible is thirty-three fold. The agglutinating power of crystalline concanavalin A is about the same as that of purified ricin or abrin. The amount extracted was estimated by comparing the ability of the extract to precipitate glycogen and to agglutinate erythrocytes with the power of a concanavalin A solution of known concentration to do likewise. It is probable that the percentage of concanavalin A varies with different crops of jack beans.

THE ISOELECTRIC POINT OF CONCANAVALIN A

Field and Teague (1907) claimed that typhoid agglutinin was electropositive and Kolmer (1924) has erroneously stated that Bechold found agglutinins to be positively charged. On the other hand, Much, Römer and Siebert (1904) found antibacterial agglutinins to be negatively charged. We have determined the isoelectric point of thrice recrystallized concanavalin A crystals

suspended in 0.1 M phosphate buffer, using the electrophoresis apparatus of Northrup and Kunitz. The crystals were found to be isoelectric at pH 5.5. Czonka, Murphy and Jones (1926), using the method of minimal solubility, found the isoelectric point of what they call "concanavalin" to be at pH 5.5.

As is well known, erythrocytes are negatively charged at neutrality and the above finding shows that the hemagglutinin must also be negatively charged at neutrality. These negative charges do not prevent agglutination; indeed, concanavalin A readily agglutinates erythrocytes from pH 5.2 to pH 7.5.

THE INACTIVATION OF CONCANAVALIN A BY ACID AND ALKALI

Goddard and Mendel (1929) observed that the ability of ricin to agglutinate erythrocytes was slowly diminished by contact with dilute hydrochloric acid. We find this to be true with concanavalin A. When dissolved in 0.1 N hydrochloric acid and allowed to stand at room temperature, the hemagglutinin irreversibly loses an appreciable amount of its ability to agglutinate, only after 2 or 3 days. The action of the acid is both reversible and irreversible. The immediate effect of the acid is to convert concanavalin A into a product which neither agglutinates erythrocytes nor gives a precipitate with glycogen and which slowly reverts after neutralization to the original concanavalin A. The reactivation requires 10 to 15 minutes at room temperature and is hastened by warming and delayed by cooling. This reversible inactivation is much more rapid than is the irreversible inactivation. The two types of inactivation by acid are shown also by crystalline urease (Sumner, Kirk and Howell, 1932) and are doubtless characteristic of proteins in general.

When dissolved in 0.1 N sodium hydroxide concanavalin A also undergoes both reversible and irreversible inactivation. It is converted reversibly into a substance which, after neutralization, will give no precipitate with glycogen, but which slowly changes back into concanavalin A. The irreversible inactivation with alkali is much more rapid than the irreversible inactivation caused by acid, and it requires only about 30 minutes at room temperature for 0.1 N alkali to destroy the hemagglutinin completely.

THE DIGESTION OF CONCANAVALIN A BY PEPSIN

If the hemagglutinin of the jack bean is identical with concanavalin A one may expect it to be inactivated by incubation with pepsin. Such has been shown to be the case.

To 200 mgm. of concanavalin A in 10 cc. of 0.1 N hydrochloric acid we added 100 mgm. of Parke-Davis 1:10,000 pepsin in 10 cc. of 0.1 N hydrochloric acid and incubated at 37°C. The inactivation was followed by testing the power of the neutralized digest, diluted 1:100, to agglutinate washed rabbit erythrocytes. The digestion of the concanavalin A was followed by testing the ability of the neutralized digest to form a precipitate with 0.2 per cent glycogen. To neutralize we first added neutral phosphate buffer and later 0.1 N sodium hydroxide in amount equivalent to the hydrochloric acid originally present. The experiment showed that the power of the neutralized digest to agglutinate and to precipitate glycogen had largely disappeared after 4 hours of incubation. On the other hand, a control containing concanavalin A in 0.1 N hydrochloric acid, although partly denatured, still possessed considerable ability to agglutinate and to precipitate glycogen.

Similar results were obtained in a second experiment where 6.8 mgm. of concanavalin A in 25 cc. of 0.05 N hydrochloric acid were treated with 1 cc. of 0.1 per cent pepsin and allowed to stand at room temperature for 48 hours. Here tests made after various intervals showed the digestion of the concanavalin A to proceed at approximately the same rate as the inactivation of the hemagglutinin.

We have attempted to digest the concanavalin A with amylase-free trypsin (Anson and Mirsky, 1933) at pH 7.0, but have found that the hemagglutinin is so resistant to tryptic digestion that it is nearly impossible to decide whether the decrease in agglutinative power is due to digestion or to spontaneous decay.

OUR METHOD FOR DETECTING AGGLUTINATION

We have employed a 2.5-per-cent suspension of thrice-washed erythrocytes in 0.85-per-cent sodium chloride containing a trace of neutral phosphate buffer. To 1 cc. of the erythrocyte suspen-

sion we have added 1 cc. of the hemagglutinin in 0.85-per-cent salt. After mixing, the test has been left either at room temperature, or at 37°C. We have noted hemagglutination by use of the microscope when it has been too incomplete to appear evident to the unaided eye. We find that the sticking of the erythrocytes to the walls of the test tube, which was used as a criterion of agglutination by Goddard and Mendel (1929), is unreliable.

THE ERYTHROCYTES OF WHAT SPECIES ARE AGGLUTINATED BY CONCANAVALIN A?

Concanavalin A agglutinates washed horse, dog, cat, rabbit, guinea pig, and rat erythrocytes in high dilution. It has a barely perceptible action upon horse erythrocytes at a final dilution of 1:10,000,000. It has no action upon well-washed human and cow erythrocytes and little or no action upon those of the goat, sheep or pig.

THE PRECIPITATION OF VARIOUS SUBSTANCES BY CONCANAVALIN A

Ricin, abrin and bean phasin (Landsteiner and Raubitschek, 1908) are known to form precipitates with the sera of various species. We have found that concanavalin A produces a precipitate with the sera of all species we have tested, although some sera give no precipitate unless a large excess of A is added. Thus, there is no relationship between the ability of concanavalin A to agglutinate the erythrocytes of a given species and the precipitation of the serum of that species with concanavalin A.

We find that serum globulins and serum mucoids are precipitated by concanavalin A. Gastric mucin, salivary mucin and egg white mucoid give precipitates. Egg yolk and baker's yeast are markedly agglutinated. If concanavalin A is added to commercial sucrase solution the yeast gum is precipitated and the sucrase is carried down.

Suspensions of corn, rice and wheat starch are well agglutinated, but potato and arrowroot starches are hardly affected. This may denote some chemical difference in the composition of the external layers of these starches, but may depend in part on the surface area of the granules. The agglutinin is absorbed readily when

shaken with rice starch, but is absorbed somewhat less readily by potato starch. Cane sugar prevents the agglutination of starch as well as the agglutination of erythrocytes.

Concanavalin A agglutinates certain bacteria, such as *Mycobacterium* and *Actinomyces*. The lipids of the paratuberculosis organism can be extracted with acetone and ether, and when suspended in salt solution the suspensions are agglutinated by A.

Concanavalin A agglutinates suspensions of jack bean, almond, olive, coconut and linseed oils, and of cocoa butter and lard. Homogenized cream is agglutinated slowly, but the agglutination is more evident after boiling, when the natural agglutinin has been destroyed. Navy bean extracts, which agglutinate erythrocytes very strongly, have no action upon fat emulsions, starch, yeast, or egg yolk.

IS CONCANAVALIN A A TOXIN?

Assmann (1911) found that extracts of the jack bean were toxic when injected into animals, but it is to be expected that the extracts employed by him contained urease. We have found previously that 0.1 mgm. of crystalline urease will always cause death if injected into a 2-kgm. rabbit. Our concanavalin solutions were quite free from urease. The intravenous injections of 1 mgm. of concanavalin A into a 2-kgm. rabbit, 2.4 mgm. into a 2.9-kgm. rabbit and 4.0 mgm. into a 3.1-kgm. rabbit caused no symptoms. The injection of 7 mgm. of concanavalin A into a 1.3-kgm. rabbit caused death in 2 days, but no intravascular agglutination was noted. The injection of 15 mgm. into a 2-kgm. rabbit caused a general intravascular agglutination of the erythrocytes. The morning after the injection the rabbit was moribund and was killed by ether.

A sheep was not affected by an intravenous injection of 5.7 mgm. of concanavalin A per kilogram. Probably this lack of toxicity was due to the resistance of sheep erythrocytes to agglutination by A. Concanavalin A is not poisonous when fed in large quantities to rabbits or to dogs.

Landsteiner and Raubitschek (1908) injected navy bean extracts into mice, rats, guinea pigs and rabbits intraperitoneally

and observed no toxic effects. From test tube experiments where we have added concanavalin A and navy bean extracts to oxalated rabbit blood we have been led to believe that the apparent non-toxicity of navy bean extracts may be due merely to their relatively weak agglutinating action and that the toxicity of concanavalin A may be credited to its intense agglutinating ability. It is possible, of course, that concanavalin A has some other action than the ability to agglutinate red cells.

THE PRECIPITATION OF GLYCOGEN BY CONCANAVALIN A

One part of glycogen in 500,000 parts of water will give a perceptible haziness if allowed to stand after the addition of excess of concanavalin A. Inasmuch as this precipitation resembles the reaction between antigen and antibody, where the glycogen takes the part of the hapten, we were interested in investigating it.

This is an instance where the quantities of each reacting substance can be measured accurately. The quantity of glycogen and concanavalin in the precipitate can be determined and also the amounts of glycogen and concanavalin A left in solution. Both glycogen and concanavalin A can be obtained in a state of considerable purity.

The precipitate formed when concanavalin A is added to glycogen is insoluble at neutrality and soluble both in acid and alkali. Crude preparations of concanavalin A contain amylase, which digests the glycogen and dissolves the precipitate, but the amylase is easily removed in our process of purifying concanavalin A.

We have added various amounts of concanavalin A solution to glycogen. The total volume of all cases has been 310 cc. After standing one-half hour or longer, the precipitate has been centrifuged off, stirred with 50 cc. of water and again centrifuged. The precipitate has been dissolved in N sulfuric acid and an aliquot taken for the determination of total nitrogen. Another aliquot has been boiled for 2 hours with a reflux condenser, cooled, neutralized exactly, and analyzed for glucose by the Somogyi-Shaffer-Hartman method (Somogyi, 1926). The factor 0.927 has been employed to calculate the glycogen. The composition of the

glycogen-concanavalin A precipitate has been found to vary somewhat, depending upon the relative quantities of concanavalin A and glycogen. This is shown in the table following:

CONCANAVALIN A AT START	GLYCOGEN AT START	A LEFT IN SOLUTION	GLYCOGEN LEFT IN SOLUTION	GLYCOGEN IN PRECIPITATE
mgm.	*mgm.*	*mgm.*	*mgm.*	*per cent*
385	188	153	0	44.5
220	188	37	0	50.8
110	207	9	0	66.4
27.5	287	5	210	79

The figures show that glycogen can be precipitated entirely by an excess of concanavalin A, but that concanavalin A dissolves in excess of glycogen.

THE MECHANISM OF HEMAGGLUTINATION

If concanavalin A is added to the laked erythrocytes of the horse, dog, rabbit, cat, rat, or guinea pig a fairly heavy precipitate is formed. On the other hand, if it is added to laked human, goat, sheep, or cow erythrocytes, no precipitate is formed. We accordingly believe that agglutination is merely a precipitation and that concanavalin A, like other hemagglutinins, unites with some chemical compound present in the surfaces of such types of erythrocytes as it agglutinates. We fully agree with the explanation which has been put forward by Eagle (1930) to explain agglutination. The ability of concanavalin A to form a precipitate with boiled starch solution and to agglutinate certain starches is in agreement with this view. The reactive compound both in the erythrocyte and in the starch granule is assumed to be a hydrophilic substance which, after uniting with the agglutinin, becomes hydrophobic. In the presence of a salt the electric charges on the hydrophobic particles are neutralized and the particles flock together and precipitate out.

We have shown by experiment that 2 cc. of washed rabbit erythrocytes when incubated with 0.7 mgm. of concanavalin A at 37°C. for 2 hours will remove the agglutinin from solution so completely that none of it can be detected in the supernatant liquid by its effect upon rabbit erythrocytes. On the other hand, under

similar conditions the non-agglutinable cow erythrocytes remove so little concanavalin A from solution that the supernatant both agglutinates rabbit erythrocytes and gives a precipitate with glycogen.

WITH WHAT SUBSTANCE IN ERYTHROCYTES DOES CONCANAVALIN A UNITE?

If rabbit erythrocytes are laked and the stromata are removed by precipitation with 1-per-cent sodium chloride, followed by centrifuging, the supernatant will give no precipitate with A, while the resuspended stromata are readily agglutinated upon adding A. It was claimed by Stillmark in 1888 that in agglutination the hemagglutinin unites with the stromata and this was confirmed by von Lieberman in 1907 and by Guyot in 1909. We have attempted to analyze the precipitate formed by precipitating laked rabbit erythrocytes with A, and have found lecithin, cholesterol, protein, purine, and carbohydrate present. We deem it possible that the chemical compound in stromata with which concanavalin A unites may be glycoprotein, but have not been able to show that it is so.

It has been stated that erythrocytes treated with formaldehyde (Guyot, 1909) are still able to agglutinate and that cow erythrocyte stromata which have been incubated with papain can still bind ricin (Jacoby, 1904–05). Coca (1908) found that horse erythrocytes after standing 15 minutes with osmic acid, with subsequent washing, could still absorb agglutinin. However, there is a distinction between binding agglutinin and possessing the ability to become agglutinated. We find that the addition of saponin or caprylic alcohol prevents rabbit stromata from agglutinating after the addition of A, and prevents the formation of a precipitate when A is added to laked erythrocytes. Heating laked erythrocytes to 37°C. causes the precipitate given with A to be more compact and less voluminous.

Stromata which have been defatted with ether or with petroleum ether can no longer be suspended in salt solution. The same applies to stromata treated with acids or heated to boiling. Rabbit stromata can be digested largely with trypsin and the material

remaining in suspension can be shown to give practically no precipitate upon adding concanavalin A.

SUMMARY

1. The preparation of concanavalin A, the hemagglutin of the jack bean, is described.

2. The isoelectric point of concanavalin A is found to be at pH 5.5, showing that the hemagglutinin is negatively charged at neutrality.

3. The hemagglutinin is both reversibly and irreversibly inactivated by 0.1 N acid and 0.1 N alkali. It is irreversibly inactivated more readily by alkali than by acid.

4. The hemagglutinin and the crystallizable protein, concanavalin A of the jack bean, are identical since concanavalin A is digested by pepsin at the same rate as that at which the hemagglutinin is inactivated.

5. Concanavalin A is toxic if a sufficiently large dose is injected into rabbits. The toxicity may be due solely to its hemagglutinating action.

6. The reaction between concanavalin A and glycogen has been studied and the precipitate formed has been found to contain from 44.5 to 79 per cent of glycogen, depending upon whether excess of A or excess of glycogen is added at the start.

7. The mechanism of agglutination is explained as a reaction between the concanavalin A and the stromata of the erythrocytes to produce a hydrophobic colloid. The charge on this hydrophobic colloid is neutralized by salts which results in the clumping of the formed elements, or agglutination.

8. Concanavalin A unites with some constituent of the stromata and, since concanavalin A unites with starch, glycogen, mucins, etc., it is possible that this may be a carbohydrate group in a protein.

NOTE: After this paper had been printed we came across a paper (White and Avery, 1913) in which it was claimed that edestin is a hemagglutinin. We have been able to demonstrate no hemagglutination with edestin solutions.

We wish to acknowledge our indebtedness to the Sage Research Fund Committee for financial aid.

REFERENCES

ANSON, M. L., AND MIRSKY, A. E. 1933 Jour. Gen. Physiol., **17**, 151.
ASSMANN, F. 1911 Arch. für die gesammte Physiol., **137**, 489.
COCA, A. F. 1908 Biochem. Zeit., **14**, 125.
CZONKA, F. A., MURPHY, J. C., AND JONES, D. B. 1926 Jour. Amer. Chem. Soc., **48**, 763, 1926.
DIXSON 1887 Australian Med. Gaz., 156, quoted from FRIEDBERGER, E., AND BROSSA, G. A. 1912 Zeit. für Immunitätsforschung und exp. Therapie, Orig., **15**, 506.
EAGLE, H. 1930 Jour. Immunol., **18**, 393.
FIELD, C. W., AND TEAGUE, O. 1907 Jour. Exper. Med., **9**, 222.
GODDARD, V. R., AND MENDEL, L. B. 1929 Jour. Biol. Chem., **82**, 447.
GUYOT, G. 1909 Centralbl. f. Bakt., Orig., **48**, 330.
JACOBY, M. 1904–5. Beiträge zur chem. Physiol., **6**, 113.
KOLMER 1924 Infection, Immunity and Biologic Therapy. 3d ed., p. 258. W. B. Saunders Company.
LANDSTEINER, K., AND RAUBITSCHEK, H. 1908 Centralbl. f. Bakt., I Abteil., Orig., **45**, 660.
MUCH, RÖMER, AND SIEBERT 1904 Zeit. für diätet. und physik. Ther., **8**, 19, 94.
SOMOGYI, M. 1926 Jour. Biol. Chem., **70**, 599.
SUMNER, J. B., AND HOWELL, S. F. 1935 Jour. Immunol., **29**, 133.
SUMNER, J. B., HOWELL, S. F., AND ZEISSIG, A. 1935 Science, **82**, 65.
SUMNER, J. B., KIRK, J. S., AND HOWELL, S. F. 1932 Jour. Biol. Chem., **98**, 543.
WHITE, B., AND AVERY, O. T. 1913 Jour. Infect. Dis., **13**, 103.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CENTRAL PENNSYLVANIA BRANCH

DANVILLE, PENNSYLVANIA, MAY 16, 1936

CLINICAL MANIFESTATIONS OF BACTERIAL SENSITIVITY. *F. W. Davison*, The George F. Geisinger Memorial Hospital, Danville, Penn.

The allergic state in the human animal has no counterpart in other animals, therefore advancement in this field must come through clinical research. An allergic individual may be described as one whose mucous membranes have a tendency to develop excessive edema in response to irritation, and it is well known that edematous tissue is favorable soil for bacterial growth. Real progress in this field can be made only after solving the problem of the mechanism of the production of this edema. Recent work has shown that in some cases endocrine deficiency or inbalance is the fundamental defect permitting the development of what is clinically recognized as the allergic state. Thyroid extract has been the means of definitely relieving some of our asthmatic patients.

Those patients who develop asthmatic symptoms following acute respiratory infection, at irregular intervals without respect to season of the year or geographical location, are allergic to the bacteria infecting either the lining of their sinuses, their bronchial mucosa, or both. This type of patient is definitely benefited by administration of autogenous vaccine. The proper dose must be determined for each individual because an over-dose promptly aggravates the symptoms. Most of these patients show no skin reaction to an intradermal injection of their autogenous vaccine. We feel that vaccines prepared by suspending the organisms in the broth in which they were grown are more effective than saline suspension vaccines. Vaccines are more effective in asthmatic children than they are in older individuals.

THE IDENTIFICATION OF BACTERIA ISOLATED FROM MILK BOTTLE CAPS. *Eleanor F. Schooley*, Bucknell University, Lewisburg, Penn.

The purpose of this study was to identify bacteria isolated from milk bottle caps. The bottle caps from which the organisms were collected were supplied by cap manufacturing companies as widely separated geographically as Missouri and Massachusetts. The bacteria found to be associated with the bottle caps were considered to carry economic significance in that the majority of them produced undesirable changes in milk.

The procedure followed was that recommended in the Manual of the Society of American Bacteriologists, with the addition of the use of blood agar as a means of distinguishing between closely related types on the basis of hemolytic action. Bergey's

Manual of Determinative Bacteriology was used as the key in the final identification of the species.

The organisms studies were all gram positive, spore-forming rods, and with few exceptions were placed in the following groups: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus vulgatus*, *Bacillus parvus*, *Bacillus globigii*, *Bacillus aterrimus*, *Bacillus graveolens*, *Bacillus mycoides*, *Bacillus petasites*, *Bacillus albolactis*, *Bacillus terminalis*, *Bacillus circulans*, and *Bacillus pseudotetanicus*. The geographical location of the manufacturing plant was found to have no significance in the types of organisms isolated.

A Preliminary Report on Antarctic Bacteria. *Chester A. Darling*, Allegheny College, Meadville, Penn.

While in the Antarctic on the Second Byrd Expedition, the chief biologist, Paul A. Siple, did some culture work with bacteria and collected various samples of snow, ice, soil, mosses, lichens, and other material which were brought to the laboratories at Allegheny College for study. Thirteen isolated cultures were brought back in sealed test tubes; in addition to these one hundred and eighty have been isolated including twelve coccus forms and about equal numbers of spore-forming and non-spore-forming rods; two cultures of yeast and nearly one hundred of molds likewise have been isolated. The cultural characteristics of these various organisms are being determined; some already identified include: *Bacillus subtilis*, *Bacillus graveolens*, *Micrococcus ureae*, and *Micrococcus candidus*.

The Value of Premedical Bacteriology. *John F. Conway, M.D.*, The George F. Geisinger Memorial Hospital, Danville, Penn.

In an attempt to determine the value of premedical training in Bacteriology, the opinions of professors of this subject in class A medical schools of the United States and Canada were solicited. A summary of these opinions follows.

Premedical education should not duplicate medical school work, and a good Bacteriology course would do just that since it is not a required course for entrance in all schools. Pathogenic Bacteriology can best be taught in correlation with clinical and autopsy material and such is not possible in most colleges. In order to satisfy medical bacteriologists the college professors must decide upon a standard course which gives the students fundamental, sound uniform training in non-pathogenic Bacteriology. Many desire this as each year it is increasingly difficult to give a reasonably complete course in medical Bacteriology in the allotted time. The next step would be the adoption of such a course as an entrance requirement to class A medical schools, so the course there would not have to be planned for students without preliminary training. In view of the many individuals who have stated that they would prefer to have students equipped with English, literature, history, philosophy, economics, embryology, etc., this would probably be difficult.

Do Vitamins Affect Bacterial Growth? *Isabel Holtzinger*, Bucknell University, Lewisburg, Penn., and The George F. Geisinger Memorial Hospital, Danville, Penn.

Heavy suspensions of bacterial cultures were streaked on the vitamin A

and vitamin D medicated agar as well as on plain extract agar, which served as control in this experiment. By studying luxuriance of growth on these media, it was found that certain vitamin concentrations definitely increased the luxuriance of growth of some bacteria, but in greater or less concentrations seemed to have no effect at all.

The most favorable concentration of vitamins was not a constant factor, but varied specifically.

Carotene, the precursor of vitamin A, definitely inhibited the growth of bacteria when used in agar in too great a concentration. However, by decreasing this concentration there was a general stimulation of growth. These results seem to indicate that bacteria are unable to fraction Carotene to vitamin A when it is present in agar in too great a concentration.

In most cases, the influence of vitamin concentrates seems to be specific for each organism as well as for each source supplying the vitamin.

However, about ninety units vitamin A in combination with approximately twelve units vitamin D per cubic centimeter of agar, seemed to give the greatest increase in luxuriance of bacterial growth. When vitamin D alone was used, there was an increase in luxuriance of growth even greater than when A and D were used in combination, indicating that vitamin A exerts inhibitory powers on bacteria.

THE SPREAD OF TULAREMIA THROUGH WATER, AS A NEW FACTOR IN ITS EPIDEMIOLOGY

S. P. KARPOFF AND N. I. ANTONOFF

Epidemiological section of the Institute of Epidemiology and Microbiology at Tomsk (Siberia); Director of the Institute, prof. G. F. Wogralik)

Received for publication April 16, 1936

Rodents are the chief sources of infection of tularemia and the reservoir of its virus in the external world. The rôle of the water-rat, which, in the U. S. S. R., is the principal source of tularemic infection for man is well known. In the United States the same part is played by the wild rabbit. In the U. S. S. R. the rôle of the hare, rabbit, and mouse,—in Norway, that of the wild rat—has been proved. Other rodents may serve as reservoirs of virus, for instance: *Apidemus silvaticus, Ondatra zibethica, Didelphis virginiana, Marmota flaviventer, Marmota bobac, Microtus ilaeus, gerbitus tamaricinus, Citellus beecheyi* Rich., *Citellus pygmeus* Pall, *Microtus californicus-aestuarinus*, etc.

Animals that do not belong to the rodent-class, such as opposums, foxes, and lemmings, have also been recorded as sources of infection.

The modes by which tularemia spreads from these sources of infection are also being carefully investigated. Contact with water-rats while hunting or flaying them has been well studied in the U. S. S. R. (Zarhy, Hatanever, Golov, Volfertz, Suvorov, Voronkova, Gauzner, Belitzer, Kniazevsky, Berdnikov, Tiflov and others). In the United States contact with wild rabbits during skinning and especially during the preparation of their carcasses is the cause of infection in 61 per cent of cases (Francis). The spread of tularemia through contact with the domestic rabbit was proved for the first time in the U. S. S. R. by Miller in collaboration with Kreutzer, Kvashnina and Grzebina. Its spread through contact with mice has also been proved by Miller con-

jointly with Volfertz, Korbon, Jampoladova and Grzebina. In this case the chief epidemiological factor is the soiling of food stuff by the excretions of the mice. The spread of tularemic infection through contact with hares, or rather with their carcasses and skins when preparing the killed hares and when making tinned food was also established first in the U. S. S. R. by Berezin and Nesgovoroff.

A number of infection-carriers or vectors have also been studied, such as mites, flies, horse-flies, fleas and water-rat lice.

Quite recently, Prof. Miller has pointed out another possible mode of spread of tularemia. In his paper on tularemia, read at the All-Russian Conference of Microbiologists and Epidemiologists, which took place in Leningrad on December 7 to 11, 1934,[1] as well as in his monograph "Tularemia," published in collaboration with Prof. Stradomsky, Prof. Miller points to water as a new possible mode of spread of tularemia. Thus, for instance, he says in his monograph "Tularemia": "Beside food stuff, water can also serve as a source of infection of tularemia. The investigations, which Dr. Somoff has carried out in my laboratory have established this fact on the ground of the study of one outbreak (the culture has been isolated from water) and of experimental data." We have no other references to this mode of spread of tularemia.

Having studied in 1935 one outbreak of tularemia we were able to prove by exhaustive evidence this new mode of spread of the disease in question. An epidemic due exclusively to water, broke out on meadows amongst people who were occupied in haymowing. In a few days, cases of disease became numerous and in a week's time nearly all the workers had been taken ill, with the exception of one group of mowers, who worked on a plot near by.

CLINICAL PICTURE

Forty-three patients only could be carefully examined and observed for some length of time. Some of the other patients had not been hospitalized at all, others had been discharged from the hospitals before our coming.

[1] This paper has been published in the Sovetskaya Vratschebnaya Gazeta, 1935, N. 3, under the title "Tularemia and new data in its epidemiology."

Disease appeared two to eight days after the people came to the meadow-lands.

All the patients noted the sudden onset of the disease with shivering on which followed a sensation of fever, headache—mostly in the frontal part and pains all over the body—pains in the back, the neck and the extremities being particularly noted. Often there was nausea, sometimes vomiting and pain in the substernal region. In some cases on the second or third day of illness there was a nasal hemorrhage. On the first day of sickness, and more often on the third to the fifth day (in some cases on the tenth day) most of the patients complained of painful swallowing. At about the same time the lymph glands behind the angle of the underjaw began to enlarge, as well as the cervical glands along the musculus sterno-cleido-mastoideus (oftener on one side than on both) and the submaxillary glands. Some patients did not show any phenomena related to the pharynx and glands and their main symptoms were fever and general malaise. In some cases inflammation of one eye was from the first a prominent feature.

The investigation of the first group of patients, some of which we were able to examine in the first days of the illness, showed the following clinical picture.

Nikita P., aged 25. Has been ill for four days.

The patient looks feverish. The temperature is 39°C. The face is hyperemic. The scleral vessels and the conjunctiva are injected. The tongue is moderately furred, moist. The appetite is reduced. Acute hyperemia of the front arches of the pharynx and the uvula. Enlargement and hyperemia of the right tonsil. Behind the angle of the underjaw on the right an enlarged lymph gland, 4 cm. of length, not adherent to the skin and the subcutaneous cellular tissue, painful at palpation. The liver protrudes from under the costal margin to a finger's breadth. The spleen begins from the eighth rib, its margin is palpable in the right-side position. Both organs are somewhat painful on palpation.

As has been stated above, we were able to see only a few patients of this group, most of the patients examined being either in the period when the illness was fully developed or in that of falling temperature. On the whole we have examined 35 patients at this stage.

The main morbid symptoms in this group of patients were the following: the temperature, which up to the seventh or tenth day of the illness ranged from 38.5° to 39.5°C., diminished after this by 1° or 1.5° and in the period from the eleventh to the twentieth day it was either normal or subfebrile in the evening. Most patients experienced severe malaise all through the illness. There was no delirium. The appetite, which had been low during the feverish period was rapidly restored after the fall of the temperature. The angina appeared, as has been stated above in some cases from the first day, more often from the third to the fifth day of illness. It was mostly of the catarrhal type and manifested itself in a hyperemia of the soft palate, the uvula and one or both tonsils.

The tonsils, especially the left one, were often enlarged and friable. Some of the patients had also follicular or lacunar angina, or an angina with small patches of fur of diphtheritic character. In three cases there was observed purulent amygdalitis of the left tonsil, with discharge into the pharynx and pus secretion. As was stated above, the lymph glands were involved in the process either behind the underjaw angle or along the musculus sterno-cleido-mastoideus and mostly on one side. If the glands along the musculus sterno-cleido-mastoideus were affected, as many as two or three glands were involved. The glands were from three to seven cm. in size. At the first examination they were never seen to adhere to the skin and the underlying tissues. In two cases we observed further a lesser mobility of the glands and the skin was then also involved in the process. Palpation of the gland began to show the presence of fluctuation and, on puncturing, a greenish pus was obtained.

Seven of the forty-three patients examined had fever and lymphadenitis in the aforementioned regions, but did not complain of pain in the pharynx—and angina was not observed either at their admittance into the hospital or later on. In five cases the chief symptom was the rise of temperature up to 38.5°C., which lasted from twelve to sixteen days. The fall of temperature was a slow lysis. As a rule there was an enlargement of the liver and the spleen. The general state of these patients was

worse than that of the patients of the two first groups. Some of them were delirious. Neither angina nor lymphadenitis were observed. One of the patients complained during the fever and for some time after the temperature had fallen of pains in the stomach region. Palpation of the stomach was painful. We could not note anything particular on palpation, although the patient remained under control for some time afterwards (Clinic of infectious diseases at the University at Tomsk).

In one case there was another prominent symptom beside fever, and that was an affection of the right eye. There was a sharp photophobia, epiphora and hyperemia of the scleral vessels and the conjunctiva. On the conjunctiva of the under lid there were several enlarged yellowish grains. These ocular phenomena disappeared in the course of the first seven days. Several days after the beginning of the ocular phenomena there appeared near the pinna an enlarged lymph gland which disappeared in the course of twenty days.

In another case, beside fever, angina and enlargement of the lymph glands behind the underjaw angle there was an affection of the left eye with the phenomena described above and also an affection of the gland near the left pinna.

In a third case also, after having suffered from shivering, heat, nausea, and pain all over the body the patient had fever with inflammatory phenomena in the left eye, enlargement of the gland near the left pinna and small ulcers under the tongue. The patient noted these ulcers on the fourth day of illness. An examination made on the seventeenth day showed that under the tongue on each side of the frenum there was a small ulcer, 1 cm. long, with a clean bottom. On touching the bottom of the ulcers with a spatula a slight sensation of pain was experienced. Examination on the thirty-sixth day of illness showed that the ulcers had disappeared. This patient had also an enlarged gland (5 to 6 cm.) on the left side under the bottom of the buccal cavity which was painful on palpation. This enlargement was observed simultaneously with the appearance of the ulcers.

In the outbreak in question morbidity did not seem to bear any relation to sex or age. With a few exceptions nearly all those

were taken ill, who had in any way been exposed to the source of infection (which will be mentioned below). No death occurred, but after the fall of temperature all the patients noted a persistent weakness and at the time of the second examination many of them were still performing only easy jobs.

This clinical picture suggested tularemia with a portal of entrance through the ring of Waldeyer and the buccal and ocular mucosa and the observed cases could be classified as belonging to five different forms of the disease. The first group must be considered as belonging to the anginal form, which takes the aspect of the bubonic form; the second group belongs to the purely bubonic form, the third to the typhoid form; while two of the patients suffered from the glandular-ocular form and two other ones showed a combined anginal and glandular-ocular form.

LABORATORY TESTS PERFORMED IN ORDER TO CONFIRM THE CLINICAL DIAGNOSIS

In order to confirm the clinical diagnosis we carried out a number of laboratory tests.

During the first examination blood was taken for the agglutination-test with *Bacterium tularense* and for the infection of guinea pigs. The blood was taken from six patients on the thirteenth to the twenty-third day of illness. In the case of five patients the agglutination test made with the killed suspension of *B. tularense*[2] was positive in a dilution from 200 to 1000 (fifteenth to twenty-third day of illness). For one patient (thirteenth day of illness) the test was negative. Guinea pigs injected intraperitoneally with the blood of these patients did not fall ill. These results are not unexpected: infection of guinea pigs with the blood of patients gives positive results if it is performed in the beginning of the illness. As to the agglutinins, they appear in the second week of the illness and in a certain percentage of cases even later.

At the same time with these tests the leucocytes and also the

[2] The suspension was prepared at the Central Public Control Institute in the name of L. N. Tarassevitsch.

Shilling's formula were determined for three of the six patients. The following results were obtained:

1. Patient V. O. (a woman). Eighteenth day of illness. Anginal form. Total 8200 leucocytes: segmented leucocytes, 50 per cent; rod-shaped ones, 4 per cent; lymphocytes, 38 per cent; monocytes, 8 per cent; no eosinophils.

2. K. O. (a woman). Twenty-third day of disease. Anginal form. Total 6800 leucocytes. Of these: segmented leucocytes, 61 per cent; rod-shaped ones, 5 per cent; lymphocytes, 32 per cent; monocytes, 1 per cent; eosinophils 1 per cent.

3. K. E. (a woman). Eighteenth day of illness. Anginal form. Total 9800 leucocytes: segmented leucocytes, 50 per cent; rod-shaped ones, 7 per cent; lymphocytes, 33 per cent; monocytes, 9 per cent; eosinophils, 1 per cent.

In three cases with severe angina the slime and fur from the tonsils were examined bacteriologically. Fuchsin staining and Giemsa staining revealed a rich flora consisting of short bacilli accumulated in heaps, small cocci, diplococci and a considerable quantity of small coccobacilli.

After the second examination on the thirty-fifth to fortieth day of illness the agglutination test was made for 21 patients. In all these cases it was positive. The dilution was 1000 to 5000. At the second examination two of the patients showed fluctuation on palpation of the enlarged lymph glands. In both cases a puncture of the gland was performed, which yielded in each case 2 cc. of greenish pus. With this pus we infected guinea pigs subcutaneously and intraperitoneally and later obtained cultures of *B. tularense*.

The clinical diagnosis of tularemia was thus confirmed serologically and bacteriologically.

EPIDEMIOLOGY OF THE OUTBREAK

The meadows on which the epidemic broke out are situated between the villages of M. and N. The country is varied in topography. There are four eminences, divided by valleys, through

which flow the river S. and three brooks, which have no particular names. One group of mowers used the water of the river S. for drinking and in this group there did not occur a single case of illness; the other mowers, amongst whom the illness rate attained 100 per cent used the water of one of the brooks, which at the time in question was running freely.

An epidemiological survey of the area was carried out after we had clinically examined the patients and diagnosed the disease. This examination was accompanied by a close questioning of the patients as well as of the managers of the hay-mowing organizations. As this questioning showed, the patients as well as the managers had observed, that all the workers, who had drunk unboiled water from the brook, were taken ill, whereas in the group of workers who used water from the river S., no cases of illness occurred. The managers of one of the mowing organizations took this fact into account and a second brigade of workers, which came to replace the group that had been taken ill, received directions to take water out of the river S. (although this was at some kilometers from their mowing place) and to boil the water before drinking. This second group finished mowing and returned to their collective farm, without having had a single case of illness, whereas in another group where these measures were not strictly followed, some of the new-coming workers were taken ill in their turn.

Questioning of the patients showed, that near the brook out of which they took their drinking-water, they had seen, as they said, some "black rats" (water rats) and had even killed one of them, but all the people who had been in the fields declared these to be but isolated examples. Beside the rats a few field mice had also been observed.

Proceeding to survey in detail the locality of the outbreak we analyzed the clinical picture of the illness as well as the data obtained from questioning and came to the definite conclusion that it was through water that the infection had been spread. The fact that such a great number of people had fallen ill in a short period of time and the presence of a definite portal of entry (tonsils, conjunctiva and buccal mucosa) from the first excluded sev-

eral of the usual vehicles of infection. There was no industry and moreover the portal of entry excludes this mode of spread. The possibility of the infection being disseminated by biting insects (such as gnats, horse-flies, flies, etc.) is also excluded by the determinate portal of entry. Spread through food we also from the first considered as impossible and the subsequent experimental and bacteriological tests have fully proved the correctness of this view. And even if we did not have at our disposal such proofs as those we have gathered, this mode of spread requires the presence of a great number of rodents, under conditions which enable them to pollute food stuffs with their fecal masses.[3] The fields in question did not contain any rodents in great quantities, this fact having been subsequently verified by our survey. Moreover, rodents would have resorted to the camp of the brigade of workers, in which there was no case of illness as well as the others and cases of illness should have occurred in this group as well.

Consequently there remains but one mode of spreading the infection, the evidence for which stands out; this is spread through water.

In surveying the meadows we paid the greatest attention to the unknown brook N., which formed the source of this infection.

This brook springs from the upper marshy part of the valley and flows into the river. The place where it empties into the river is covered with thick vegetation and is hardly to be noticed from the opposite side of the river. All along the brook, which measures about 2.5 km., its banks are thickly grown with birches, aspens, bird-cherry trees, bushy willows and some needle-trees and currant bushes. In some places there are footpaths in the thick grass on which the workers used to go and fetch water. The brook is only 5 to 10 cm. deep and its bottom is covered with crushed stone and pebbles. At the places at which water had been drawn, there are artificial holes, up to 0.5 m. deep. The water in the brook is good flowing water, at places one hears its murmur. Neither fish nor other cold-blooded animals could be observed. Nor could we notice any rats or other animals, though

[3] Prof. Miller points to house-mice, but in this case even field-mice could scarcely have played that part.

in several places we saw otter-holes with fresh tracks leading to them.

LABORATORY TESTS OF WATER

In order to test the etiological rôle of water in spreading the infection, we injected samples of this water into guinea pigs on the thirty-first day after the beginning of the epidemic. We took some water for bacteriological and chemical analysis and for the purpose of testing it as to the presence of *B. tularense*. The temperature of the water measured in the brook on a cloudy day was 15°C. The water for bacteriological and chemical analysis was taken at the place where the drinking water had been drawn.

The following data were obtained from analysis:

The water was colorless and inodorous, with a slight flaky brown-red sediment. The reaction was alkaline. Oxidization—12 mgm. for 1 liter. Traces of ammonia and nitrous acid. Nitric acid, sulphuric acid, Cl and Fe are not found. General hardness, 14.5; removable, 14.0; constant hardness, 0.5. Dense sediment, 20.0 mgm. The bacteriological test showed the presence of a considerable quantity of microörganisms (up to 2000 colonies in 1 cc.) and many anaerobes. No intestinal bacilli were found in 10 cc.

Both the chemical and the bacteriological analyses suggested that the water was somewhat polluted.

In order to reveal the presence of *B. tularense* we immediately injected samples of the water into guinea pigs which we had brought with us. The water was injected subcutaneously into five guinea pigs. Each of the pigs received 10 cc. of water. On the next day all the guinea pigs showed infiltration at the site of injection. On our return to the Institute we noted that the guinea pigs had become noticeably thinner (they had not been weighed before being injected as our work was carried out under field conditions). On the ninth to the twelfth day all the guinea pigs died. Three or four days before death all the guinea pigs showed the presence of a number of enlarged lymph glands in the hypodermic tissue of the abdominal wall, in the form of "beads"

running from the site of injection to one or both groins. Autopsy revealed the presence of pathologico-anatomical changes in the organs of the guinea pigs as are characteristic for tularemic infection.

As an example of this we are giving here the shortened autopsy record of one of the guinea pigs.

Guinea pig 3. (Autopsy performed by the veterinarian consultant of the Institute, docent Urborsky.)

Water was injected on August 20. Death on August 29. Autopsy on August 29. The hair is easily pulled out. The hypodermic tissue is feebly pronounced. The visible mucosae are pale, dry, somewhat cyanotic. The lymph glands of the thoracic region are enlarged (one above the ensisternum on the left, the other beneath it on the right). Both glands are as large as a middle-sized pea. On palpation they are thick, on section they show a yellowish color and stratified consistency. The glands are but slightly adherent to the underlying hypodermic tissue. In the region of the white abdominal line the lymph glands, seven in number, are lentil-sized and are disposed in the form of a string of beads. Groin glands—one on each side, the right one as large as a middle-sized bean, the left one lentil-sized, brown-red on section. In the peritoneal cavity there is some bloody liquid. The mesenteric glands are slightly enlarged, juicy, of a dark brown color. The spleen is 4 by 2 by 0.4 cm., of brown-red color. The capsule is strained. Under it there are multiple, scattered, small, limited whitish-yellow foci, of the size of a poppy-seed or larger. On the surface of the porta of spleen there are similar foci. The consistency is thick and brittle. The surface of the section shows the same foci, of a grayish-yellow color. The liver is considerably enlarged, brown-red. The lobulation is levelled. The right lobule has one large hempseed-sized focus and a multitude of small grayish-yellow foci, as large as a poppy-seed or smaller. The left and middle lobules show the same changes. On section there are to be seen disseminated foci of the same color. The left renal capsule is somewhat enlarged, the left one normal in size. The limit between the cortical layer and the medullar one is pronounced. On being sectioned these layers show three yellow white foci of a thick, crumby consistency as large as a millet-seed. The bladder mucosa shows injected vessels and hemorrhagic dots. The findings in the other organs being normal, we do not mention them here.

These extracts from the autopsy record show a pathological-anatomical picture characteristic for tularemic infection as regards the lymphatic system, the spleen, the liver and the renal capsules. I. V. Toropzev, assistant in pathological anatomy in the Medical Institute at Tomsk, made histo-pathological preparations from all these organs as well as from the heart and the kidneys. (We take this opportunity of thanking him.)

The microscopy of the preparations showed the following data:

Lymph gland. Focal infiltration, developing in some places into suppurative liquefaction, with a pronounced reaction of the reticular cells and of the cells of the endothelium. The inflammatory process is not only localized in the parenchyma of the nodule, but goes over to the nodular capsule. Consequently, there is an acute periadenitis.

Spleen. Acute inflammatory hyperplasia. The organ is affected by a focal leucocyte infiltration, which in some places develops into abscesses.

Liver. Focal necroses with a pronounced inflammatory reaction on the periphery of the foci. Suppurative infiltration round the central veins developing here and into abscesses.

Renal capsules. Phenomena of fatty degeneration in the cells of the organ.

Kidneys. Congested plethora of the glomerular capillaries and degenerative changes in the epithelium of the tubules accompanied by phenomena of karyolosis.

Heart. Degenerative changes of albuminous character in the parenchyma.

From the liver, the spleen and the lymph glands of guinea pigs, which had died after having been injected with 10 cc. of water, we prepared emulsions and with these emulsions six guinea pigs were injected subcutaneously and intraperitoneally. The guinea pigs which had received the emulsion intraperitoneally, died on the third day, those which had been infected subcutaneously, showed a gradual loss of weight and died on the fifth day.

The necropsy of the pigs which had died from intraperitoneal infection revealed in all cases an enlargement of the groin glands and of the mesenteric glands; the changes in spleen and liver

(enlargement of the organs and presence of foci) were, quantitatively speaking, much less pronounced than in the case of pigs infected subcutaneously. Phenomena of peritonitis, perihepatitis and perisplenitis were to be observed in all the cases (autopsy records are not quoted in order not to lengthen this paper). The necropsy of the pigs infected subcutaneously with emulsions from the organs shows changes in the lymphatic system, the spleen and the liver which are as pronounced as in the case of the guinea pigs which had died from an injection of water (see autopsy record quoted above). In the case of the emulsion infection, however, considerably greater changes were to be observed in the mesenteric glands.

From the liver, the spleen and the lymph glands of the guinea pigs which had died from water injection and from these same organs of guinea pigs, which had died of an emulsion injection of organs (liver, spleen and lymph glands) taken from pigs infected with water injection, sowings were made on the medium of McCoy and that of Chapin. Smears were prepared and stained after Giemsa. The microscopy of the smears showed an enormous quantity of small bacilli, mostly in the form of coccobacilli. At places the microörganisms form a thick layer. For the most part they are free, but some are included in the leucocyte protoplasma.

On the media of McCoy and Chapin there could be observed, after an interval of forty-eight to seventy-two hours (rarely after twenty-four hours) a slight hardly noticeable growth of non-confluent colonies. After prolonged gram-staining, the culture showed gram-negative coccobacilli and short bacilli.

In order to test the specificity of the isolated cultures they were injected into guinea pigs subcutaneously and intraperitoneally at the rate of 0.2 cc. of culture from a test-tube. All the guinea pigs showed phenomena of diffuse peritonitis and died twenty-four to forty-eight hours after the intraperitoneal infection. Some of them showed such changes in the glands and the liver as are characteristic for the tularemic microörganism. The following short autopsy findings may illustrate this fact.

The guinea pig 11 was injected on August 31 intraperitoneally

with 0.2 cc. of the emulsion of the pure culture 3. Died on September 2.

On the abdomen, to the right of the white line the lymph gland is pea-sized. To the left there are two lentil-sized glands. Enlargement of the inguinal and the mesenteric glands. Perihepatitis, perisplenitis (the surface of the liver and the spleen covered with fibrin clots and grayish white films, which are easily taken off). The liver is enlarged. On its left lobe there are two small, white-yellow foci. On the middle-lobe there are several small foci. The spleen is enlarged but without any noticeable changes.

The guinea pigs infected subcutaneously with pure culture all died on the fifth day after emaciation and characteristic changes in the lymph glands, the liver and the spleen. This may be illustrated by extracts from one of the autopsy records.

The guinea pig 15 received on August 31 0.2 cc. of pure culture emulsion subcutaneously. Died on September 5. It had grown very thin. Weight before infection, 500 grams; on the day of the death, 370 grams.

At the site of injection there is a considerable infiltration, 5 by 4 cm. large. On both sides of the abdominal line, the lymph glands are enlarged; they are bean-sized on the right side and pea-sized on the left. The liver is enlarged and has on its left lobe a large compact focus, which fills nearly one third of the lobe. Section shows a grayish-yellow color. When scraped the surface of the cut crumbs easily. On the right and the middle lobes of the liver there are eight foci, ranging in dimensions from the size of a millet seed to that of a lentil, of the same color and consistence as the foci on the left lobe. The spleen is 4 by 2 by 0.4 cm. large. Its borders are rounded, the capsule is strained. The surface of the organ is covered with a multitude of small foci, as large as poppy seeds. Owing to the great quantity of these foci, the spleen has a grayish-white color. A section of the spleen shows that its pulp is also penetrated by these foci.

These records show, that intraperitoneal injection leads to death from peritonitis. The subcutaneous injection of the culture gives a characteristic pathological-anatomical picture.

From the organs of the guinea pigs which had died from infection with pure culture we obtained *B. tularense*.

All the isolated cultures (19 strains)—those obtained from guinea pigs which had died from the injection of water, as well as those cultivated from the organs of guinea pigs which had died after being injected with emulsion of the first pigs, were agglutinated with five sera of patients from one of the collective farms. All the cultures showed a pronounced agglutination with all the sera in dilutions from 1500 to 2000.

All these data definitely prove the etiology of the isolated cultures, and their relation to the described outbreak.

TESTING OF PATHOLOGICAL MATERIAL FOR THE PRESENCE OF B. TULARENSE

Glandular punctures were made on two patients (patient O. on the thirty-eighth day of illness and patient T. on the thirty-sixth day); and with the pus of each of the patients we infected two guinea pigs. Out of the four infected guinea pigs two (one of each pair) died; one on the eighth day, the other on the thirteenth. The necropsy revealed characteristic pathological-anatomical changes and from the liver of both guinea pigs we obtained cultures of *B. tularense*. Both the patients in question were placed in the Clinic for infectious diseases of the Medical Institute at Tomsk (Director prof. G. F. Wogralik) and on the fifty-second and fifty-fourth day of illness another glandular puncture was performed. Pus was obtained, in one case (patient O.) 4 cc., considerably mixed with blood, in the other (patient T.) 17.5 cc. The pus of the latter patient was thick and had a greenish color. The pus of patient O. was injected in equal parts into two guinea pigs and another pair of pigs was infected with the pus of patient T. at the rate of 5 cc. each. The infection was performed subcutaneously and intraperitoneally. One of the pigs infected with the pus of patient O. died on the tenth day, and one of those infected with the pus of patient T. died on the twelfth day. The necropsy revealed pathological-anatomical changes characteristic for infection with *B. tularense*.

These data show that in suppurated but not discharging

lymphatic glands *B. tularense* can maintain its vitality for a long period.

The study of the outbreak does not end here, as some links are still lacking in the epidemiological evidence. At present investigation is being carried on, in order to discover the source of infection of the described outbreak. A number of important data are already available, but the studies being not complete, we shall not dwell upon this question for the present.

Our observations lead us to the following conclusions:

1. In the outbreak in question it was the tonsils, the mouth, the mucosa and the conjunctiva that formed the portal of entrance of infection.

2. In this outbreak the anginal form was predominating and, the typhoid form came next while a number of patients showed the glandular-ocular form of illness.

3. We did not observe any correlation between morbidity and sex or age.

4. A new mode of spread of tularemia was proved experimentally and bacteriologically, i.e., its spread by water.

5. The quantity of microörganisms in the infected water courses may sometimes be so considerable, that their isolation does not meet with great difficulties.

6. In our experiments 100 per cent of the guinea pigs infected with the water died and their organs showed characteristic pathological-anatomical changes. From the organs of all these guinea pigs by water we obtained cultures of *B. tularense*.

7. The cultures that were isolated from water did not differ in regard to virulence from those that were isolated from the suppurated lymph glands of the patients. When these cultures were tested on guinea pigs, death followed in a 100 per cent of cases and the organs showed changes characteristic of tularemic infection.

THE OCCURRENCE OF SALMONELLA, ORANIENBURG TYPE, IN AN INFECTION OF QUAIL[1]

PHILIP R. EDWARDS

Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Kentucky

Received for publication April 24, 1936

In October, 1935, the writer received from Dr. Robert Graham an unidentified organism of the *Salmonella* group which had been isolated from an epizootic disease of baby quail. Dr. Graham, to whom the writer is indebted for a description of the outbreak of disease, stated: "Culture 1035 was isolated on plain agar plates by seeding liver tissue of quail that died in large numbers on our state quail farm. The loss occurred over a period of several weeks and in birds from three days to one month of age. About sixty percent of the baby quail succumbed." The purpose of this paper is to report the identification of the organism involved.

MATERIALS AND METHODS

The cultures used in the work were as follows:

Quail 1035—Organism isolated from baby quail. Received from Dr. Robert Graham.

Suipestifer 16—*Salmonella suipestifer* 16. Received from Dr. L. F. Rettger. Originally from the collection of Dr. T. Smith.

Kunzendorf 1713—*Salmonella* (Kunzendorf) 1713, Nat'l Coll. Type Cultures, London.

Oranienburg 3746—*Salmonella* (Oranienburg) 3746, Nat'l Coll. Type Cultures, London.

Berlin 2988—*Salmonella* (Berlin) 3078, Nat'l Coll. Type Cultures, London.

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by the permission of the director.

Potsdam 9781—*Salmonella* (Potsdam) 3026 Nat'l Coll. Type Cultures, London.

Bareilly 3213—*Salmonella* (Bareilly) 3213 Nat'l Coll. Type Cultures, London.

Virchow 3748—*Salmonella* (Virchow) 3748 Nat'l Coll. Type Cultures, London.

Jena 1891—*Salmonella enteritidis* var. Jena 1891—From Dr. F. Kauffmann.

Moscow 3205—*Salmonella enteritidis* var. Moscow 3205 Nat'l Coll. Type Cultures, London.

Rostock 3747—*Salmonella enteritidis* var. Rostock 3747 Nat'l Coll. Type Cultures, London.

Dublin 2206—*Salmonella enteritidis* var. Dublin 2206A Nat'l Coll. Type Cultures, London.

In performing agglutination tests for somatic antigens, the bacillary suspensions were prepared by the method of Gardner (1929). The tests were incubated at 52°C. for four hours and held in the ice box over night before reading. In preparing flagellar antigens, infusion broth cultures were incubated at 37°C. for 15 to 17 hours and diluted with an equal amount of saline containing 0.5 per cent formalin. The tests for floccular agglutination were read after four hours incubation at 52°C. In absorbing flagellar agglutinins the serums were treated with the growth of 60 plate cultures per 1 cc. of undiluted serum. In absorbing somatic agglutinins the growth from 30 plate cultures per 1 cc. of undiluted serum was used.

RESULTS

Examination of the culture revealed that it possessed the cultural and biochemical properties characteristic of the genus *Salmonella*. The organism produced acid and gas from glucose, mannitol, maltose, arabinose, dulcitol, sorbitol, rhamnose, xylose and trehalose. Inositol was not attacked. The organism produced hydrogen sulfide and displayed active motility in broth cultures.

Somatic antigens. An antigen prepared from the quail bacillus by the method of Gardner (1929) was agglutinated in high dilu-

tion by antiserums derived from *S. suipestifer* and the Kunzendorf type. Serums representative of the other somatic groups did not cause agglutination in significant dilutions. An antiserum prepared with the organism from quail agglutinated alcohol-treated antigens of *S. suipestifer* and the Kunzendorf type to the granular titer of the serum. Reciprocal absorption tests resulted in a complete removal of somatic agglutinins from *S. suipestifer* serum by the quail bacillus and a total exhaustion of somatic agglutinins from the serum of the quail bacillus by *S. suipestifer*. It was obvious that the organism was a member of group C of the Kauffmann-White classification.

Flagellar antigens. The results obtained in the study of flagellar antigens are given in table 1. It is evident that the organism isolated from quail occurs only in the specific phase. Group components are entirely absent as shown by the failure of its serum to agglutinate suspensions of the Kunzendorf and Berlin types. This serum also failed to agglutinate non-specific suspensions of *Salmonella aertrycke* and *Salmonella anatum*. Likewise the quail bacillus was not agglutinated by non-specific serums of *S. aertrycke*, *S. anatum* and the Kunzendorf type.

The only organism of the *S. suipestifer* group which was agglutinated to any extent by quail 1035 antiserum was the Oranienburg type. This organism reacted to the titer of the serum. Likewise the bacillus from quail was agglutinated to titer by Oranienburg antiserum. Among the variants of *S. enteritidis* only the Jena type reacted in significant dilution to quail 1035 antiserum. Further, the Jena type failed to remove agglutinins for the Oranienburg type from quail 1035 antiserum. These reactions indicated that the organism from quail possessed antigenic factors m and t. The mutual absorption of agglutinins by quail 1035 and Oranienburg confirmed this and established the culture from quail as a member of the Oranienburg type, having the antigenic formula VI, VII: m, t:−.

The Oranienburg type of *Salmonella* was first isolated by Kauffmann (1930) from the stools of an apparently normal inmate of an institution where an outbreak of diarrhea had occurred. Strains received by Kauffmann from Prof. W. Rimpau and from

TABLE 1

Flagellar antigens of quail 1035

	SERUMS											
	Quail 1035	Oranienburg 3746	Quail 1035 absorbed by Rostock 3747	Oranienburg 3746 absorbed by Rostock 3747	Quail 1035 absorbed by Moscow 3205	Oranienburg 3746 absorbed by Moscow 3205	Quail 1035 absorbed by Dublin 2206	Oranienburg 3746 absorbed by Dublin 2206	Quail 1035 absorbed by Jena 1981	Oranienburg 3946 absorbed by Jena 1981	Quail 1035 absorbed by Oranienburg 3746	Oranienburg 3746 absorbed by Quail 1035
Quail 1035	20,000	40,000	20,000	20,000	20,000	20,000	20,000	20,000	10,000	20,000	0	0
Oranienburg 3746	20,000	40,000	20,000	20,000	20,000	40,000	20,000	20,000	10,000	20,000	0	0
Suipestifer 16	0	0	0	0	0	0	0	0	0	0	0	0
Kunzendorf 1713	0	0	0	0	0	0	0	0	0	0	0	0
Berlin 2988	0	0	0	0	0	0	0	0	0	0	0	0
Potsdam 9781	0	100	0	0	0	0	0	0	0	0	0	0
Bareilly 3213	0	0	0	0	0	0	0	0	0	0	0	0
Virchow 3748	100	0	0	0	0	0	0	0	0	0	0	0
Jena 1891	2,000	5,000	1,000	5,000	1,000	2,000	1,000	2,000	0	0	0	0
Moscow 3205	100	100	0	0	0	0	0	0	0	0	0	0
Rostock 3747	0	0	0	0	0	0	0	0	0	0	0	0
Dublin 2206	100	100	0	0	0	0	0	0	0	0	0	0

Figures indicate highest dilution at which agglutination occurred.
0 indicates no agglutination at 1 to 100.

Dr. K. W. Clauberg also belonged to this type. The latter cultures were isolated from healthy persons and from cases of gastroenteritis in human beings. These cultures were later described by Rimpau and Steinert (1930) and by Seligmann and Clauberg (1931). Kauffmann (1936) reported the repeated isolation of Oranienburg bacilli from the feces of two persons in Copenhagen.

The identification of the organism from quail as the Oranienburg type constitutes the first report of the presence of this bacillus in America. It is also the first record of the appearance of this type in diseases of animals.

SUMMARY

An organism occurring in a disease of baby quail was identified as *Salmonella*, type Oranienburg. This report constitutes the first recognized occurrence of this type in diseases of animals.

REFERENCES

GARDNER, A. D. 1929 Jour. Hyg., **28**: 376.
KAUFFMANN, F. 1930 Zeitschr. f. Hyg., **111**: 221.
KAUFFMANN, F. 1936 Personal communication.
RIMPAU, W., AND STEINERT, K. 1930 Münch. med. Wchnschr., 1570.
SELIGMANN, E., AND CLAUBERG, K. W. 1931 Zeitschr. f. Hyg., **112**: 254.

STUDIES OF FRESHWATER BACTERIA

III. QUANTITATIVE ASPECTS OF THE DIRECT MICROSCOPIC METHOD[1]

ARTHUR T. HENRICI

Laboratories of Bacteriology at the University of Minnesota, Minneapolis, and at Station-on-Alexander, Cushing, Minnesota

Received for publication April 4, 1936

In the first paper of this series (Henrici, 1933), there was described a procedure for studying the distribution of bacteria in freshwater habitats, which consisted in immersing microscopic slides in water for a period of time, removing them, and examining the stained preparations under the microscope. It was proposed that this method might be used quantitatively to determine microbic activity in a given habitat, the number of bacteria deposited upon a unit area of glass in a unit period of time serving as a measure of this activity. However, this measure cannot be so used until something is known about its limitations and reliability, and until certain factors, such as optimum duration of immersion, area of slide to be examined, etc., have been determined. The present paper deals with an inquiry into these matters.

A study of these slides presents a problem somewhat similar to the ideal ecological problem, the population of an island suddenly upthrust in the ocean. The immersion of the slide at once creates a new habitat, a surface upon which sessile organisms may grow; and it is not long before this habitat is found by such organisms. There develops rapidly a population characteristic of the particular part of the lake in which the slide has been put down. As in the population of other habitats, the coating of the

[1] Aided by grants from the Graduate School of the University of Minnesota, and from the National Research Council.

submerged glass proceeds in successions, certain groups of microbes appearing early, to be followed and to some extent replaced by other types. Presumably an equilibrium will eventually be established in a climactic flora and fauna.

First to come, are the minute bacteria, which may produce an appreciable coating within a few hours in the warmer waters of a shallow bay. These are shortly followed by various algae, mostly diatoms in the spring and fall, dominantly green in the summer. Sessile types of protozoa, such as *Vorticella* and the Suctoria, appear later, and the rich grazing ground afforded by the bacteria and algae attracts numerous other types of protozoa. Amoebae and creeping ciliates travel over the surface, destroying as they go. Finally, pond snails may cut wide swaths with their rasping jaws, leaving a trail of amorphous slime; leeches find the slides a convenient resting place to wait for their prey, and various invertebrates come to lay their eggs.

The quantitative analysis of such a population is a problem of some complexity. Considering the bacteria alone, the numbers found will depend in part upon the numbers brought to the slide either by water currents or by their own motility. This is probably determined entirely by chance, and depends upon the density of the bacteria in the water, the area of slide exposed, and the duration of immersion. These bacteria, however, also grow upon the slide. This is the most important feature of the method. It selects those bacteria which are active in the habitat, and ignores those which are dormant. The rate of growth will vary not only with environmental conditions, but also with different species. It is highly probable that many of these bacteria arrive at the slide as swarmers, and that they undergo a marked transformation upon becoming sessile. Certain types appear only after a prolonged period of immersion, and probably pass through a long lag phase before developing upon the slide. Finally, the numbers found will be greatly affected by the destructive action of protozoa, and there is also some evidence that the growth of algae tends to reduce the numbers of bacteria. After a time the bacteria will begin to die and disintegrate, and there may possibly be another factor involved, namely a loosening and sloughing off

of large masses of growth similar to the "unloading" of the coating on rocks in sewage filters. A consideration of all these factors indicates that nothing may be surmised *a priori* regarding the reliability of the method or the optimum duration of exposure, but that these must be determined empirically.

The method described was already in use by Karsinkin before the publication of our first paper, but his publication was delayed until a year later (Karsinkin, 1934). He refers to the bacteria which grow upon submerged slides as the bacterial "periphyton," applying a term introduced by Behning (1928) to include all aquatic organisms growing upon submerged surfaces, i.e., an ecologic group in addition to and distinct from the plankton (floating organisms) and the benthos (bottom organisms). This seems to be a useful term, and preferable to the "attachment bacteria" used by ZoBell and Allen (1933). We shall refer to bacteria which grow attached to submerged surfaces as "periphytic bacteria," and to the growth itself as bacterial "periphyton."

Karsinkin (1934) studied the distribution of periphytic bacteria in Lake Glubokoje, where previously Duplakoff (1933) had made an extensive study of the growth of the nonbacterial periphyton on glass slides. Slides were suspended at depths varying to 15 meters, those at the deepest levels resting partly in the bottom mud. They were stained with 5 per cent erythrosin, and 40 fields were counted on each slide, the results being expressed as bacteria per square centimeter. Slides were observed after immersion for periods of 1, 3, 7, 14, 24, and 28 days.

The observations of longer duration yielded the surprising result that in general fewer bacteria were found than with shorter periods. These results are attributed to the presence of algae and protozoa on the slides immersed for longer periods, which are absent on the slides left in the water for less than 3 days. Karsinkin believes that many of the bacteria originally populating the slide die and disintegrate; this in fact could be observed after 3 days.

Using the data from short duration observations at the beginning of September, when the lake was thermally stratified, the distribution of the bacterial periphyton showed a good correspondence with the values for temperature and for dissolved oxygen. Observations made at the end of September, when the lake was approaching thermal homogeneity, showed greatly reduced numbers of bacteria and a rather uniform distribution from top to bottom. Slides immersed partly in the bottom

mud showed a marked difference, both quantitative and qualitative, between the portion which was buried in the mud and the portion which was in the water immediately above, a much larger number of bacteria appearing below the mud line.

In a second paper (Karsinkin and Kusnetzowa, 1934), the method was used in a study of polluted waters. Again, 40 fields were counted. Duplicate slides did not show a variation of more than 10 per cent from the mean. It was found that in these polluted waters a short immersion (12 to 24 hours) gave more reliable results. The counts varied with the temperature, and in colder waters a longer exposure was necessary. The bacterial counts on these slides correlated with the degree of pollution as determined by chemical analyses, and with the numbers of colonies developing when duplicate slides were imbedded in nutrient agar. Colon bacilli did not grow upon the slides.

ZoBell and Allen (1933) applied the method to a study of the rôle of bacteria in marine fouling, and found that bacteria were the first organisms to appear on surfaces submerged in sea water. They found that 24 of 73 pure cultures isolated from sea water were capable of growing upon a glass surface, and correctly point out that the method selects from the habitat only certain species of bacteria capable of such attachment. In a second paper (ZoBell and Allen, 1935) further details of technique are given, and the types of marine periphytic bacteria are discussed. It is believed that the slimy film of bacteria is an important factor in the fouling of ships' bottoms.

This procedure has now been used during two seasons in a study of the distribution of the bacterial periphyton in Lake Alexander, a shallow eutrophic lake in central Minnesota, and during one season in a study of various lakes, shallow and deep, oligotrophic, dystrophic and eutrophic, in northeastern Wisconsin. While the analyses of these data have not been completed, enough has been worked through to allow of some evaluation of the method. It was early found that the technique as originally described required some modification, the most important change being a substitution of 50 x 75 mm. slides for the standard 25 x 75 mm. size. This is necessary because for some reason the organisms tend to become more densely deposited at the edge of the slide, and there is an area 5 to 10 mm. wide along the edges where the deposit shows a gradation from the edge toward the center.

With the larger slides one may ignore this border zone and confine the observation to an area in the center where the distribution is more uniform.

In counting, each cell has been recorded as a unit; if filamentous and articulated, each element has been counted; continuous filaments have been counted as one. All counts have been made with a 90× objective and 7× focussing ocular containing a ruled counting disc. The area of the counted field is 0.007744 sq. mm. In the data presented in this paper the counts are given as bacteria per field.

It was believed that a count of less than 50 fields would not be sufficiently representative, and that a count of more than 100 fields would be too laborious to be practical. It was decided therefore, to count 50 fields from each slide, and if the distribution seemed irregular, to count an additional 50 fields. There was no arbitrary rule for this, the decision being left to the judgment of the technician doing the counting. The fields were spaced by means of a graduated mechanical stage over an area approximately 20 x 40 mm. in the center of the slide.

The following data have been compiled from 620 slides put down in Lake Alexander at various stations, in varying depths, at different seasons, and for varying periods of time, and are probably fairly representative of the entire range of quantitative results which may be expected from the method. There were 380 slides on which 100 fields were counted. These ranged in mean number per field from 1.6 to 173, with an average value of 28. The coefficient of variation ranged from 24 to 323, with an average value of 81. There were 240 slides on which 50 fields were counted. The mean number per field varied from 12 to 542, with an average of 174. The coefficients of variation ranged from 7 to 71, the average being 37.

The limits recorded indicate the extreme limits of practical counting. With numbers ranging in the neighborhood of 1 or 2 cells per field the counts are quite unreliable, while with an excess of 500 per field the cells are too closely crowded to be clearly distinguished or accurately counted. The rather high variation in the distribution of the bacteria on the slides is probably charac-

teristic, and dependent on the scattered occurrence of the organisms in the water. A single cell attaching itself to the glass may multiply rapidly and produce a field filled with bacteria, while many adjacent fields will be empty. Thus, the longer the duration, the greater will be the chance for a particular area of glass to become populated. But, since the rate of increase on the slides varies markedly with different stations, there is no apparent negative correlation between the coefficients of variation and the duration of exposure. There is, however, a strong negative correlation between the coefficients of variation and the actual counts, as may be seen from an inspection of the graph shown in figure 1. As the mean number per field increases, the variation in distribution decreases, reaching a minimum in the neighborhood of 100 cells per field, beyond which there is no marked improvement with increasing counts.

The reliability of the method may be tested by a consideration of replicate slides from the same habitat. Two hundred pairs of slides have been studied with regard to their correspondence in counts. These slides were fastened back to back, and suspended from a wire in the water. They were placed in various stations in Lake Alexander, and are also representative of nearly the whole range of conditions which may be expected. The means of the counts of the two slides varied from 3.1 bacteria per field to 447. The average deviations for these 200 pairs ranged from 0.11 to 75 per cent, with a mean value of 12.31 per cent. The ratio of the difference between the two counts to the standard error of the difference varied from 0.04 to 11.22, with an average of 2.35. In 58 of the 200 cases this ratio exceeded the conventional value of 3 which is assumed to indicate significance. That is, in about one-fourth of the cases where two slides were exposed for the same time back to back, the differences in the counts are of such an order as to indicate the possibility of an environmental difference between the two sides. That such an environmental difference may occur is also indicated by observations of the kinds of bacteria present. Not infrequently a morphologically peculiar organism has been found abundantly on one of a pair of slides, completely lacking on its duplicate. Possibly water currents or differences in light intensity are involved. In any

case, there is a clear indication for the use of two slides in each observation, to be fastened back to back.

In 29 observations 2 pairs of slides, or 4 replicates, have been counted. These counts were made to obtain the values for the first 5 days in some of the time curves shown in figure 2. They are not, therefore, representative, since they include only slides

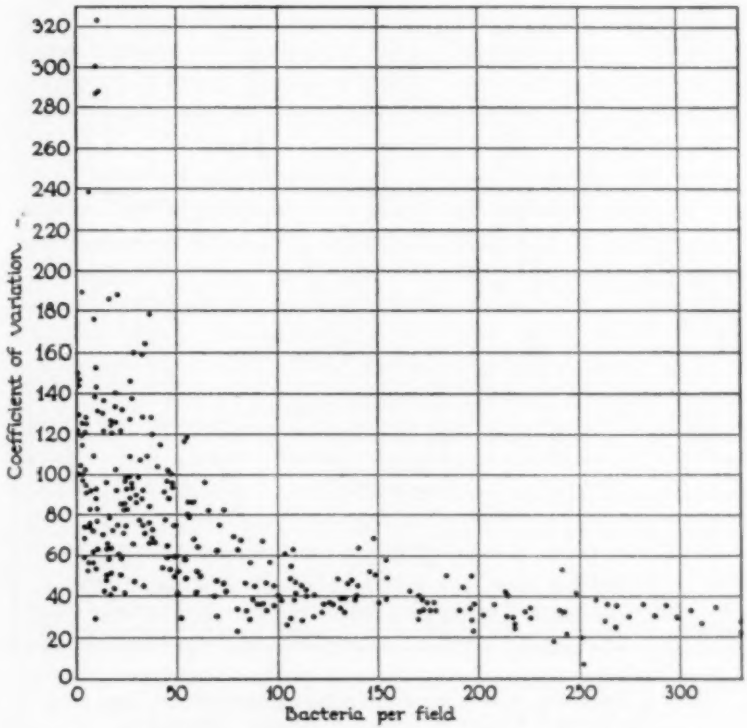


FIG. 1. RELATION BETWEEN MICROSCOPIC COUNTS AND DISPERSION

with relatively low counts. The counts ranged from 3.9 to 294 bacteria per field. The average deviations ranged from 2 to 51 per cent, with a mean value of 15.1 per cent. One set of 10 replicates from a shallow bay in Lake Alexander, with a mean count of 132 bacteria per field, gave an average deviation of 16 per cent.

A consideration of these data may lead to some pessimism regarding the quantitative use of the method, on the grounds of inaccuracy. The method is, however, almost as reliable as other quantitative bacteriological techniques; the coefficients of variation are of the same order as those encountered in Breed counts of milk, or in other microscopic counting processes, while the

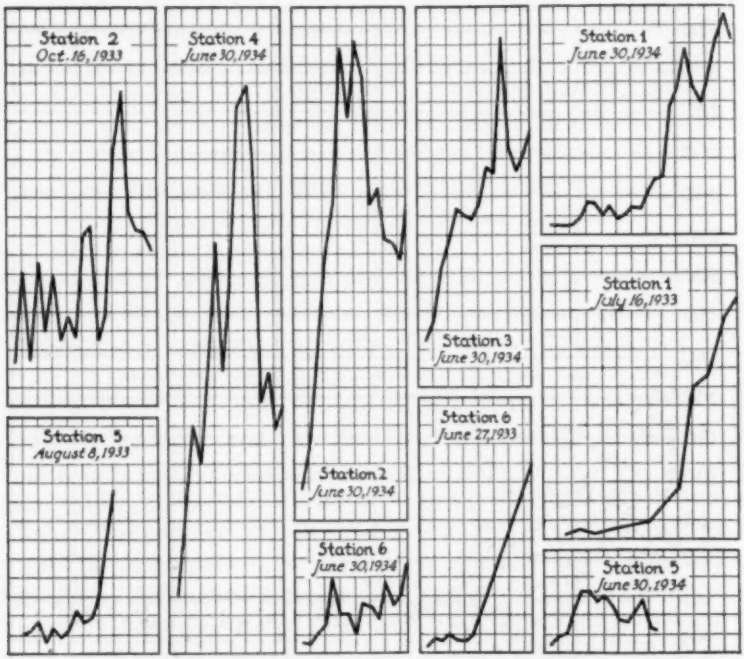


FIG. 2. RELATION OF NUMBER OF BACTERIA DEPOSITED TO TIME OF EXPOSURE. ABSCISSAE, 1 UNIT = 2 DAYS. ORDINATES, 1 UNIT = 15 BACTERIA PER FIELD

average deviations are of the same order as those met in making replicate plate counts from soil or water. The reliability of the method cannot be estimated without considering the range of values to be measured; a yardstick may be as useful as a micrometer for some sorts of measurement. At Station 4 in Lake Alexander, a warm, shallow weed-choked bay, an average count

of over 60 bacteria per field has been obtained from a single exposure for 24 hours; while in the cold deep hypolimnion of Trout Lake in Wisconsin, the bacteria are too few to count after 30 days. This is the extreme in difference that has been encountered so far. Between these limits every gradation occurs, and it is obvious that the microscopic count, inaccurate as it is, may be used to measure these differences, so long as the limitations are kept in mind and the procedure is not used to measure too finely. From the information at hand it would seem likely that a mere doubling of numbers in two observations will not be significant, but that a tenfold difference will probably mean something.

In order to obtain some information regarding the proper length of time to expose the slides, a number of them have been put down simultaneously in various stations in Lake Alexander, and removed at daily, or longer, intervals. These were all placed near the surface, suspended from the under sides of paraffined wooden floats. The counts from these slides are presented graphically in figure 2. The curves are rather irregular, as might be expected from what has already been learned regarding the variability of the counts. Part of this irregularity, however, is due to environmental factors. Surface water may change very rapidly in temperature with changes in the weather or direction of the wind, and conditions are by no means uniform through prolonged periods of exposure of the slides.

A striking feature of these curves is the difference of form when the various stations are compared. Stations 2, 3 and 4 are shallow bays with submerged aquatic plants growing in the immediate vicinity. Each has a depth of about 1 meter. Station 1 is situated in the open lake, the depth being 7 meters, with no bottom plants. Station 5 is in the middle of a small sheltered bay, 2 meters deep, with *Ceratophyllum* growing on the bottom but not reaching to the surface. Station 6 is a wave-swept rocky reef, the water 0.5 meter deep at the point where the slides were placed.

The curves tend to fall into 2 groups. Those for stations 2, 3 and 4 are similar, and may be considered characteristic of the

lenitic habitats. The bacteria develop with great rapidity, shortly reach a peak and begin rapidly to decrease. The bacteria which grow here are mostly non-filamentous types. The curves for station 1 show 2 phases, an initial period of slow increase extending for about 2 weeks, during which the organisms are mostly haplobacteria, followed by a period of more rapid development, the new organisms being mainly filamentous types (trichobacteria). It is probable that the latter require a long lag period during which they undergo some sort of transformation before becoming capable of growing as periphytic microbes. Stations 5 and 6 may be considered as being transitional between the preceding 2 groups.

A consideration of these curves indicates that if the method is to be used quantitatively the time of immersion must be adjusted, at least roughly, to the habitat. Since the time relationship is not a straight line, and varies with different habitats, a better measure would be obtained if the slides could be immersed until they reached equal numbers, comparing the duration of immersion, rather than comparing counts on slides immersed for equal lengths of time. This is of course not entirely practical, but may be roughly approximated. It was found that greater accuracy was obtained if the counts reached 100 per field or more. If the slides are left in the water until they become visibly frosted over and feel slimy to the touch, they are approaching this condition. In some stations the counts will, however, never reach such a value.

It is interesting to compare the data from which these curves were drawn in another way, to note the mean increase per day on slides immersed in the same habitat for varying lengths of time. These values are presented in table 1. It will be seen that in some cases there is a tendency for the bacteria to be deposited at a rate which is roughly constant over a considerable number of days. In each series the average deviations from the mean of the series has been computed. These may be compared with the deviations of replicate slides immersed for a uniform period, which have been given previously, and may serve as a measure of the amount of error introduced by varying the time of exposure

of the slides. This error is not so great as might have been expected from the curves. It would seem that, if the duration

TABLE 1

Relation of microscopic counts to duration of exposure of slides

Bacteria per field per day of immersion

DAYS	STATION 1, JULY 16, 1933	STATION 1, JUNE 30, 1934	STATION 2, JULY 14, 1933	STATION 2, OCTOBER 16, 1933	STATION 2, JUNE 30, 1934	STATION 3, JUNE 30, 1934	STATION 4, JUNE 30, 1934	STATION 5, AUGUST 8, 1933	STATION 5, JUNE 30, 1934	STATION 6, JUNE 27, 1933	STATION 6, JUNE 30, 1934
1		8.2	35	35	26	20	46		7.1	4.1	8.6
2		3.9		53	30	18	61	7.9	6.9	5.9	4.3
3	0.9	2.1	24	12	43	25	60	5.9	5.2	3.4	5.0
4		2.1		28	52	28	38	6.3	8.3	3.8	5.7
5	1.2	2.8	30	12	50	25	44	1.6	9.6	2.4	12
6		4.2	19	17	62	20	54	3.5	8.0	1.5	10
7	2.6	3.5	14	7.7	46	17	32	1.8	5.8	1.8	4.4
8		2.1	17	9.0	47	17	37	2.2	5.8		1.7
9	0.7	2.5	18	6.9	34	18	48	3.8	4.3		4.5
10		1.4	17	14	25	16	45	2.3	2.9		4.0
11		1.4	18	13	24	24	35	2.3	2.4		2.5
12	0.8	1.7	15	4.4	19	14	17	3.9	2.7		4.8
13		1.6		5.7	17	12	17	8.8	3.2		3.0
14	0.9	2.4	14	15	15	12	13		1.4		3.4
15		2.9		17	17	13	13		1.2	9.8	4.7
16	1.7	2.9		4.8							
17		6.0		8.2							
18	2.3	6.6		7.7							
19		7.2		6.5							
20	6.7	5.9									
21		5.0									
22	6.0	5.7									
23		6.7									
24	7.8	7.2									
25		6.2									
26	7.4										
Mean	3.1	4.1	20	15	34	19	37	3.9	5.2	4.1	5.2
Deviation	79%	46%	26%	56%	38%	23%	35%	41%	42%	52%	45%

of exposure is roughly adjusted to the habitat by inspection of the slides, and if two time intervals be used in each observation,

the variations in the time curves do not introduce enough error to invalidate the use of the method.

It is to be emphasized that the microscopic method selects only those bacteria in the water capable of growing attached to a submerged surface. Plate cultures select those organisms capable of growing upon the medium used which happened to be present in the water at the time the sample was taken. Undoubtedly plate cultures include a great many organisms which are not characteristic of the water, which are not growing in it, but which have been washed in from the surrounding soil or have dropped in from the air. Nevertheless one may assume that in a general way the numbers of bacteria of all kinds will vary with the suitability of the habitat for life, and might therefore predict a degree of correlation between the microscopic counts and plate counts.

A number of plate counts have been made at the same stations and at the same depths, and approximately contemporaneously with the exposure of slides, which may be used to calculate a coefficient of correlation between the two methods of measurement. In some cases two or more plate counts were made during the time the slides were in the water; the results of these have been averaged. Conversely, in several instances samples were taken for plating on a day when one set of slides was taken up and another put down; here the microscopic counts have been averaged. The plating medium contained sodium caseinate 0.05 per cent, peptone 0.05 per cent, glycerol 0.05 per cent, starch 0.05 per cent, dibasic potassium phosphate 0.05 per cent and agar 1.5 per cent. Flat-sided narrow-mouth bottles were used for plating, and 5 replicate cultures were made from each dilution. These were incubated for 2 to 3 weeks at room temperature. The observations have been made at all stations in Lake Alexander and in 9 different lakes in northeastern Wisconsin.

There are 100 pairs of counts which may be used. The plate counts ranged from 1 to 50,420 per cubic centimeter, the mean being 328. The microscopic counts ranged from 0.1 to 37 bacteria per field per day, the mean being 4.92. The Pearsonian coefficient of correlation, r, calculated from these counts, was 0.625 ± 0.041. This was surprisingly high. The distribution

of both sets of counts was badly skewed, but the range of the plate counts was considerably greater than that of the slide counts, and graphing did not indicate any clear trend. It was thought therefore that the Pearsonian coefficient might have indicated a spurious correlation. Recalculating by the method of rank differences gave a coefficient of 0.606. It is therefore concluded that this coefficient is valid and that there is a limited positive correlation between the two sets of measurements.

This correlation is not, however, an indication that the two methods may be used interchangeably. It merely shows that, through a wide range of habitats, conditions which are suitable for one group of bacteria may also be suitable for another. Perhaps any other measure of life, as total plankton, organic

TABLE 2

Comparison of plate counts with microscopic counts

	STATION 1	STATION 2	STATION 3	STATION 4	STATION 5	STATION 6	STATION 7
Number of samples plated	28	6	6	6	10	8	7
Mean number of bacteria per cubic centimeter	928	8,701	10,224	28,554	3,189	1,927	901
Number of microscopic counts	80	9	16	16	28	7	24
Mean number of bacteria per field per day	3.5	18.7	9.8	26.5	3.3	4.9	3.9

matter in the water, or number of bottom plants, would give a similar correlation. But if studies are limited to more restricted habitats, the correlation disappears. This may be illustrated by individual observations.

In table 2 there are presented the averages of a number of plate counts and of microscopic counts from 7 different stations in Lake Alexander. These stations have been already described, save for station 7, which is an open lake station near the deepest part, 13 meters deep. It will be seen that, while there is not a precise rank order correlation, the stations tend to group in the same manner by both methods. The lenitic stations give higher plate counts and also higher microscopic counts.

But when we make a comparison of different depths at the

same station, the superiority of the microscopic method as an indicator of bacterial activity becomes apparent. Such observations are presented in table 3. In each case the observations have been made in the open lake, at or near the deepest part. Lake

TABLE 3

Comparison of bacterial plankton with bacterial periphyton in various types of lakes

LAKE ALEXANDER

Depth	Temperature August 22, 1933	Plate count, September 11, 1933	Slide count, July 29 to August 22, 1933
meters			
0	25.0	276	Too many
2			5.6
3		515	8.9
4			1.8
5		210	1.1
6			2.5
7		56	3.9
8			5.2
9			4.7
10			3.9
11			1.8
12		738	0.9
13	21.0		2.1

MARY LAKE

Depth	Temperature July 10, 1935	Plate count, July 24, 1935	Slide count, July 10 to August 8, 1935
meters			
0	25	65	1.4
2	14	25	0.2
4	5.3	17	Too few
6	4.1	29	Too few
8	4.0	15	Too few
10	4.0	16	Too few
12	4.0	19	Too few
14	4.0	20	Too few
16	4.0	25	Too few
18	4.0	26	Too few
20	4.3	41	Too few

TROUT LAKE

Depth	Temperature, August 21, 1935	Plate count, July 15, 1935	Slide count, July 22 to August 21, 1935
meters			
0	23	64	0.9
2	23	34	0.7
4	22	59	1.9
6	22	65	0.7
8	22	72	1.3
10	16	62	1.5
12	13	45	0.03
14	11	55	0.02
16	8.8	70	Too few
18	7.9	8	Too few
20	7.5	9	Too few
22	7.2	12	Too few
24	7.0	35	Too few
26	6.8		Too few
28	6.4	58	Too few
32	6.3	124	Too few

HELMET LAKE

Depth	Temperature, July 9, 1935	Plate count, July 9, 1935	Slide count, July 9 to July 25, 1935
meters			
0	24	44	14
2	16	58	0.4
4	9.1	91	0.4
6	6.6	130	Too few
8	6.4	110	Too few
10	6.3	160	Too few

Plate counts = bacteria per cubic centimeter of water. Slide counts = bacteria per field per day.

Alexander is relatively shallow, with a broad surface exposed to the prairie winds. It does not, therefore, become thermally stratified in the summer. The variation in temperature from top to bottom recorded in the table is the greatest that has been observed, and the water is probably fairly uniform throughout both in temperature and in chemical composition. Except for the very heavy coating of the surface slides, due more to algae than to bacteria, which made a count impossible, there is no significant variation in the vertical distribution of the bacteria as measured by either method.

Trout Lake is a deep clear, oligotrophic lake with a very definite thermal stratification, the thermocline occurring at about 12 meters. Although there is a slight decrease in the plate counts from samples below the thermocline, this difference is not marked and is within the errors of the method. But the microscopic counts show a very definite drop. In those cases marked "too few," only a very occasional cell was found in many fields; the stained slides looked like clean glass. After 30 days exposure the slides above the thermocline showed an average of 35 cells per field, those below the thermocline practically none.

Mary Lake and Helmet Lake are dystrophic, fed by dark water from sphagnum bogs. Mary Lake is a narrow deep pool in a spruce forest, and shows an extremely rapid drop in temperature, light intensity, and oxygen below the surface. This change is reflected in the slide counts, not in the plate counts. Helmet Lake is similar but shallower. There was a tremendous growth of of bacteria at the very surface, none below. The plate counts show a progressive increase toward the bottom, probably due to sedimentation. Undoubtedly the majority of the bacteria growing on these plates had been washed in from the surrounding bog lands.

SUMMARY

The use of a direct microscopic method for measuring bacterial activity in freshwater habitats is discussed; statistical and other errors and the limitations of accuracy, are pointed out. The relationship of the number of bacteria to the duration of exposure of the slides has been studied. It is found that the curves for

this relationship vary with, and are probably characteristic of, different habitats. There has been found a limited positive correlation between plate counts and microscopic counts. Reasons for this correlation and its limitation are discussed.

It is recommended that if the method is used quantitatively, each observation be made on 2 slides 50 x 75 mm., to be suspended in the water back to back; that the duration of exposure be roughly adjusted to the habitat, and that two observations of differing duration be made for each habitat, trying for a count of 100 or more per field (roughly 13,000 per sq. mm.); and that 50 fields be counted from each slide, from an area in the central portion.

The author is indebted to Miss Lucretia Wilder (deceased), to Miss Delia Johnson, and to Mr. Graham Smith for their patient and painstaking work in making the slide counts and in computing the data; and to Miss Elizabeth McCoy, Mr. Fred Williams, and Mr. Newell Sjolander for assistance with the observations on Wisconsin lakes, and for some of the plate counts used.

REFERENCES

Behning, A. 1928 Das Leben der Wolga. Bd. V. Die Binnengewässer. E. Schweizebart, Stuttgart.

Duplakoff, S. 1933 Arb. der Limnolog. Station zu Kossino, **16**, 1–160. (Russian with German summary.)

Henrici, A. 1933. Jour. Bact., **25**, 227–286.

Karsinkin, G. 1934. Arb. der Limnolog. Station zu Kossino, **17**, 21–48. (Russian with German summary.)

Karsínkin, G., and Kusnetzowa, Z. 1934. Arb. der Limnolog. Station zu Kossino, **18**, 91–107. (Russian with German summary.)

ZoBell, C., and Allen, E. 1933 Proc. Soc. Exp. Biol. and Med., **30**, 1409–1411.

ZoBell, C., and Allen, E. 1935. Jour. Bact., **29**, 239–251.

THE ACTION OF HEXAMETHYLENETETRAMINE ON MEMBERS OF THE COLON AND AEROGENES GROUPS

CHARLES F. POE AND J. HOWARD WILLIAMSON

Division of Sanitary Chemistry, University of Colorado, Boulder, Colorado

Received for publication April 27, 1936

The presence of members of the aerogenes (*Aerobacter*) group of bacteria offers considerable interference in the positive identification of the members of the colon (*Escherichia*) group in waters. Many attempts have been made by research workers to find selective media by which the members of the above-mentioned groups might be differentiated. One of the most recent selective media for such a differentiation has been proposed by Wilson (1933), who used a broth containing hexamethylenetetramine (urotropin). This author claims that urotropin medium has the power of inhibiting strains of the *Aerobacter* group and, at the same time, of allowing the growth of the members of the *Escherichia* group.

The following quotation from the publication of Wilson is of interest:

> In hexamine I discovered that I had a substance which in a certain concentration allowed a rich growth of *B. coli* and completely arrested the growth of *B. lactis-aerogenes*.
>
> From many experiments, I arrived at the following simple selective medium: 1000 cc. of water, 10 grams peptone, 5 grams lactose, and 20 cc. of 40 per cent solution of hexamine (hexamethylenetetramine). The peptone lactose water is sterilized, and to it, when cool, the hexamine solution is added. . . .
>
> It is interesting to note that hexamine itself, apart from liberating formaldehyde, has an antiseptic action, and that this is very marked with strains of *B. lactis-aerogenes*. . . . My conclusion as to the selective action of hexamine in the above medium is based on the study of 101

strains of *B. coli* isolated from separate samples of human faeces, 11 from urine, 27 from water, 6 from cow's milk, and 10 from the excreta of cattle.

Wilson also proposes a solid enrichment medium which will cause the suppression of members of the aerogenes group and permit the growth of members of the colon group.

Ohira (1920), Hanzlik and Collins (1914), and Trendelenburg (1919) found that urotropin itself had no bactericidal power. Milanesi (1927), on the other hand, found this substance in certain concentrations to inhibit *Eberthella typhosa* and *Escherichia coli*.

The authors of this paper decided to make use of the media proposed by Wilson for testing a rather large collection of bacteria belonging to the *Aerobacter* and *Escherichia* groups.

Jordan (1911), and Hanzlik and Collins (1914), state that urotropin will liberate formaldehyde in acid solutions. We prepared our medium according to the directions given by Wilson except that the urotropin was added before sterilization, the reaction of the medium having previously been made slightly alkaline (pH 7.2) in order to prevent the formation of formaldehyde. This medium, in test tubes containing 10 cc. each, was sterilized in an Arnold sterilizer for 30 minutes on each of three successive days.

Actively growing cultures (transferred daily for five days into fresh peptone broth) of the members of the *Aerobacter* and *Escherichia* groups were inoculated into the medium. In no case was growth noted in any of the tubes after three days.

Media were then prepared in which the amount of urotropin was progressively decreased from 800 mgm. per 100 cc. Preliminary tests were made in order to determine what amounts of urotropin in the media would permit growth. This amount was found to be about 6 mgm. to 100 cc. Different lots of media were then prepared containing from 2 to 12 mgm. of urotropin to each 100 cc. After being tubed, each concentration of medium was divided into two lots. One half was sterilized in the Arnold sterilizer as before, and the other half was sterilized in the autoclave at a pressure of 15 pounds for twenty minutes.

Twenty members each of the colon and aerogenes groups were inoculated into each of the two lots of media. These experiments were repeated on freshly prepared media, and the average results are recorded in tables 1 and 2. Throughout all experiments the temperature of incubation was 37°C.

An examination of tables 1 and 2 indicates that there is little difference in the inhibitive action of hexamethylenetetramine towards the members of the *Aerobacter* and *Escherichia* groups.

TABLE 1

Growth of E. coli and A. aerogenes in hexamethylenetetramine media

Media sterilized in the Arnold

NUMBER OF CULTURES USED	0.02*	0.04	0.05	0.06	0.08	0.10	0.12
			E. coli				
3	+†	−	−	−	−	−	−
3	+	+	−	−	−	−	−
7	+	+	+	−	−	−	−
5	+	+	+	+	−	−	−
2	+	+	+	+	+	−	−
			A. aerogenes				
3	+	−	−	−	−	−	−
5	+	+	−	−	−	−	−
3	+	+	+	−	−	−	−
5	+	+	+	+	−	−	−
2	+	+	+	+	+	−	−
2	+	+	+	+	+	+	−

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

If anything, the members of the latter group are more susceptible to the urotropin than the members of the *Aerobacter* group, this condition being contrary to that reported by Wilson. The media sterilized in the Arnold proved to be more toxic than those which were sterilized in the autoclave. In the former case, media containing from 0.06 to 0.08 mgm. per cubic centimeter inhibited growth for the majority of the organisms, and in the latter case the amounts were from 0.08 to 0.10 mgm. as compared to 8 mgm.

in the experiment of Wilson. The media sterilized by each method were tested for formaldehyde, and this substance was found to be present in both sets of media. Cazzani (1929) and Thomann (1930) have reported that urotropin in solution decomposes when heated.

The formation of formaldehyde would account for at least a part of the antiseptic action of the media. The preparation of the media was then somewhat modified. The basic lactose

TABLE 2

Growth of E. coli and A. aerogenes in hexamethylenetetramine media

Media sterilized in the autoclave

NUMBER OF CULTURES USED	0.02*	0.04	0.05	0.06	0.08	0.10	0.12
E. coli							
1	+†	+	−	−	−	−	−
2	+	+	+	−	−	−	−
9	+	+	+	+	−	−	−
6	+	+	+	+	+	−	−
2	+	+	+	+	+	+	−
A. aerogenes							
3	+	+	+	−	−	−	−
8	+	+	+	+	−	−	−
7	+	+	+	+	+	−	−
2	+	+	+	+	+	+	−

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

medium without the urotropin was sterilized in the autoclave for twenty minutes at 15 pounds. The urotropin was precipitated from alcohol and dried; and weighed amounts were placed in sterile flasks. These flasks were then exposed to dry heat for one hour at 110°C. The sterilized lactose broth was added to the flasks containing the various amounts of urotropin so that the strengths of the latter substance varied from 4 to 26 mgm. per cubic centimeter. The media were tubed aseptically, and allowed to stand 7 days at room temperature in order that any acci-

dental contamination might be detected. This procedure eliminated the heating of the urotropin solution, and the consequent formation of formaldehyde was thought to be avoided. However, when 35 members each of the colon and aerogenes groups were inoculated into this medium, the results were of the same

TABLE 3

Growth of members of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Media stood seven days

NUMBER OF CULTURES USED	2*	4	6	8	10	12	14	16	18
Escherichia group									
2	+†	−	−	−	−	−	−	−	−
5	+	+	−	−	−	−	−	−	−
5	+	+	+	−	−	−	−	−	−
10	+	+	+	+	−	−	−	−	−
8	+	+	+	+	+	−	−	−	−
3	+	+	+	+	+	+	−	−	−
1	+	+	+	+	+	+	+	−	−
1	+	+	+	+	+	+	+	+	−
Aerobacter group									
6	+	+	−	−	−	−	−	−	−
2	+	+	+	−	−	−	−	−	−
1	+	+	+	+	−	−	−	−	−
6	+	+	+	+	+	−	−	−	−
6	+	+	+	+	+	+	−	−	−
7	+	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	+	+	+

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

order as those found before, except that the organisms grew in a greater concentration of urotropin. The average results of two runs are listed in table 3. On the average, the greater number of the colon organisms failed to grow in concentrations around 10 mgm.; whereas most of the members of the aerogenes group grew in concentrations up to about 14 mgm.

Since the media had stood for seven days, it was assumed that formaldehyde had been produced. Tests for this substance confirmed the assumption.

Similar lots of media varying in strength of urotropin from 4 to 30 mgm. per cubic centimeter were prepared. After being tubed

TABLE 4

Growth of members of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Media stood one day

NUMBER OF CULTURES USED	8*	10	12	14	16	18	20	25	30
Escherichia group									
2	+†	−	−	−	−	−	−	−	−
1	+	+	−	−	−	−	−	−	−
1	+	+	+	−	−	−	−	−	−
1	+	+	+	+	−	−	−	−	−
3	+	+	+	+	+	−	−	−	−
5	+	+	+	+	+	+	−	−	−
11	+	+	+	+	+	+	+	−	−
7	+	+	+	+	+	+	+	+	−
4	+	+	+	+	+	+	+	+	+
Aerobacter group									
1	+	−	−	−	−	−	−	−	−
1	+	+	−	−	−	−	−	−	−
5	+	+	+	−	−	−	−	−	−
1	+	+	+	+	−	−	−	−	−
1	+	+	+	+	+	−	−	−	−
2	+	+	+	+	+	+	−	−	−
2	+	+	+	+	+	+	+	−	−
7	+	+	+	+	+	+	+	+	−
15	+	+	+	+	+	+	+	+	+

* Milligrams of hexamethylenetetramine per cubic centimeter.
† + = growth; − = no growth.

aseptically, these media were allowed to stand until the next day when they were inoculated with the same organisms used to obtain the results in table 3. The average results obtained with media prepared at two different times are recorded in table 4.

In the media inoculated after standing for one day, the organisms were able to grow in a much higher concentration of urotropin that those inoculated into the medium which stood seven days.

TABLE 5

Growth of members of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Media stored at 4°C.

HOURS MEDIA STOOD AT 4°C.	2*	4	8	12	16	20	24	28	32	40	50
Escherichia group (average of 5)											
0	+†	+	+	+	+	+	+	+	+	+	−
24	+	+	+	+	+	+	+	+	+	+	−
48	+	+	+	+	+	+	+	+	+	−	−
96	+	+	+	+	+	+	+	+	+	−	−
144	+	+	+	+	+	+	+	+	−	−	−
216	+	+	+	+	+	+	+	−	−	−	−
288	+	+	+	+	+	+	−	−	−	−	−
480	+	+	+	+	−	−	−	−	−	−	−
624	+	+	+	−	−	−	−	−	−	−	−
792	+	+	−	−	−	−	−	−	−	−	−
Aerobacter group (average of 5)											
0	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	−
48	+	+	+	+	+	+	+	+	+	−	−
96	+	+	+	+	+	+	+	+	+	−	−
144	+	+	+	+	+	+	+	+	−	−	−
216	+	+	+	+	+	+	+	−	−	−	−
288	+	+	+	+	+	+	−	−	−	−	−
480	+	+	+	+	+	−	−	−	−	−	−
624	+	+	+	−	−	−	−	−	−	−	−
792	+	−	−	−	−	−	−	−	−	−	−

* Milligrams of hexamethylenetetramine per cubic centimeter.
† + = growth; − = no growth.

Four cultures of the colon group and 15 cultures of the aerogenes group were able to grow in concentrations of 30 mgm. per cubic centimeter. When the media stood for seven days, none of the organisms grew in media containing more than 18 mgm. Tests

for formaldehyde were made on the media which stood for one day, and only a very slight indication of its presence was noted.

Experiments were next conducted on the urotropin media after standing for various lengths of time at 4°, 20°, and 37°C. Inocu-

TABLE 6

Growth of members of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Media stored at 20°C.

HOURS MEDIA STOOD AT 20°C.	2*	4	8	12	16	20	24	28	32	40	50
Escherichia group (average of 5)											
0	+†	+	+	+	+	+	+	+	+	−	−
24	+	+	+	+	+	+	+	−	−	−	−
48	+	+	+	+	+	+	−	−	−	−	−
72	+	+	+	+	+	−	−	−	−	−	−
96	+	+	+	+	−	−	−	−	−	−	−
144	+	+	+	−	−	−	−	−	−	−	−
216	+	+	−	−	−	−	−	−	−	−	−
264	+	−	−	−	−	−	−	−	−	−	−
312	−	−	−	−	−	−	−	−	−	−	−
Aerobacter group (average of 5)											
0	+	+	+	+	+	+	+	+	+	+	−
24	+	+	+	+	+	+	+	+	−	−	−
48	+	+	+	+	+	+	+	−	−	−	−
72	+	+	+	+	+	+	−	−	−	−	−
96	+	+	+	+	+	−	−	−	−	−	−
144	+	+	+	+	−	−	−	−	−	−	−
216.	+	+	−	−	−	−	−	−	−	−	−
264	+	−	−	−	−	−	−	−	−	−	−
312	−	−	−	−	−	−	−	−	−	−	−

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

lations were made into each lot of the media at various intervals with five members each of the colon and aerogenes groups. Check determinations were made with freshly prepared media. Inoculations were made every twenty-four hours for the first ten days; every 48 hours for the next ten days; and thereafter every

seventy-two hours. The results of a number of the inoculations are recorded in tables 5, 6, and 7.

A study of the last three tables clearly shows that the media became more toxic with the passage of time and at higher temperatures. Tests for formaldehyde showed increasing amounts of this substance as the toxicity increased.

TABLE 7

Growth of members of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Media stored at 37°C.

HOURS MEDIA STOOD AT 37°C.	2*	4	8	12	16	20	24	28	32	40	50
Escherichia group (average of 5)											
0	+†	+	+	+	+	+	+	+	+	+	−
12	+	+	+	+	+	+	+	−	−	−	−
24	+	+	+	+	−	−	−	−	−	−	−
48	+	+	+	−	−	−	−	−	−	−	−
72	+	+	+	−	−	−	−	−	−	−	−
96	+	−	−	−	−	−	−	−	−	−	−
120	−	−	−	−	−	−	−	−	−	−	−
144	−	−	−	−	−	−	−	−	−	−	−
Aerobacter group (average of 5)											
0	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	−	−	−
24	+	+	+	+	+	−	−	−	−	−	−
48	+	+	+	−	−	−	−	−	−	−	−
72	+	+	−	−	−	−	−	−	−	−	−
96	+	−	−	−	−	−	−	−	−	−	−
120	−	−	−	−	−	−	−	−	−	−	−
144	−	−	−	−	−	−	−	−	−	−	−

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

Fifty members each of the colon and aerogenes groups were selected to be inoculated into media immediately after the tubing of the solutions. The members of the *Escherichia* group included *E. coli, E. paragruenthali, E. formica, E. communior, E. pseudocoloides, E. anindolica,* and *E. neapolitana.* The members of the

Aerobacter group included *A. aerogenes*, *A. oxytocum*, *A. cloacae*, *A. hibernicum*, and *A. levans*. The different species of the *Escherichia* and *Aerobacter* groups showed only slight differences in ability to grow in the different media. The results are, therefore, consolidated in table 8.

TABLE 8

Growth of 50 additional members each of the Escherichia and Aerobacter groups in hexamethylenetetramine media

Inoculated immediately after preparation

NUMBER OF CULTURES USED	5*	12	16	20	24	30	40	50	60
Escherichia group									
2	+†	−	−	−	−	−	−	−	−
1	+	+	−	−	−	−	−	−	−
7	+	+	+	−	−	−	−	−	−
9	+	+	+	+	−	−	−	−	−
11	+	+	+	+	+	−	−	−	−
12	+	+	+	+	+	+	−	−	−
7	+	+	+	+	+	+	+	−	−
1	+	+	+	+	+	+	+	+	−
Aerobacter group									
2	+	+	−	−	−	−	−	−	−
1	+	+	+	−	−	−	−	−	−
6	+	+	+	+	−	−	−	−	−
12	+	+	+	+	+	−	−	−	−
14	+	+	+	+	+	+	−	−	−
8	+	+	+	+	+	+	+	−	−
6	+	+	+	+	+	+	+	+	−
1	+	+	+	+	+	+	+	+	+

* Milligrams of hexamethylenetetramine per cubic centimeter.

† + = growth; − = no growth.

The results in table 8 indicate that the different organisms vary greatly in their ability to grow in urotropin media. The majority of the organisms grew well in media containing 25 mgm. or more per cubic centimeter. A number of organisms grew in media containing as much as 50 mgm. of urotropin per cubic centimeter.

A series of experiments were tried with solid media containing

urotropin. The composition of the medium follows: 1 gram peptone, 0.5 gram lactose, 2 gram Bacto-agar, water 100 cc. To separate sterile amounts of this basic agar medium, various amounts of sterile urotropin crystals were added so as to make the urotropin content from 3 to 40 mgm. per cubic centimeter. The media were then poured into petri dishes.

Actively growing cultures of the members of the colon and aerogenes groups were then streaked on these different media. Results similar to those observed with the liquid media were obtained except that it took lesser amounts of urotropin to inhibit growth on the solid media than in the liquid media. The medium cannot be used, as suggested by Wilson, to differentiate the members of the colon and aerogenes groups. Members of the aerogenes group, on the whole, grow better than did members of the colon group.

CONCLUSIONS

The following conclusions may be drawn from this study of the growth of the *Escherichia* and *Aerobacter* groups in urotropin-media.

1. The members of the *Aerobacter* group grow in liquid media containing a greater concentration of urotropin than do members of the *Escherichia* group. The media cannot be used to differentiate the two groups as claimed by Wilson.

2. Urotropin media become more toxic with passage of time and at higher temperature because of the production of formaldehyde.

3. Solid media containing urotropin also fail to differentiate between the *Escherichia* and *Aerobacter* groups of bacteria.

REFERENCES

CAZZANI, U. 1929. Boll. chim. farm., **48**, 821; C. A., **24**, 467.
HANZLIK, P. J., AND COLLINS, R. J. 1913. Arch. Intern. Med., **12**, 578; C. A., **8**, 753.
JORDAN, A. 1911. Biochem. Jour., **5**, 274.
MILANESI, E. 1927. Arch. farmacol. sper., **43**, 205; C. A., **22**, 608.
OHIRA, T. 1920. Centr. Bakt. Parasit., Abt. I, **85**, 63; C. A., **15**, 1163.
THOMANN, J. 1930. Pharm. Acta. Helv., **5**, 30; C. A., **24**, 5110.
TRENDELENBURG, P. 1919. Biochem. Zeit., **95**, 146; C. A., **14**, 932.
WILSON, J. D. 1933. Jour. Hyg., **33**, 404.

AN ATYPICAL MENINGOCOCCUS ISOLATED FROM CEREBROSPINAL FLUID IN THE CALCUTTA MENINGITIS EPIDEMIC OF 1934–35

BENODE BEHARI SEN

Director, Serum Institute of India (Biological Laboratory, Union Drug Company, Ltd., Calcutta, India)

Received for publication May 2, 1936

INTRODUCTION

Between the years 1932 and 1935 there were frequent outbreaks of meningitis in an epidemic form in Calcutta and disappointing results were obtained with serum therapy in Calcutta hospitals. The same disappointing results were also reported from the United States of America. Sara Branham of the National Institute of Health, United States Public Health Service, Washington, D. C., took up the study of the meningococci involved in the outbreaks there, to find out if the strains involved might be different from those found in earlier epidemics. She typed about 375 strains of meningococci and concluded that "On the whole, the meningococci found during the recent epidemics are not essentially different from those found in 1915–18, in the sense that except for 14 strains, which seemed to be a different species, they could be placed in the same serological group" (Branham, 1932). "The remaining strains (14 strains) did not fall into any of these four serological groups represented in the Gordon-Murray classification" (Branham, 1930), and their "colonies on blood agar are less moist than those of meningococci, they produce a golden yellow pigment and *do not* ferment any of the carbohydrates generally used in classifying this genus, viz., dextrose, levulose, maltose and sucrose." This is the description of a new meningococcus-like organism isolated from the cerebrospinal fluid by Sara Branham and has since been known as *Neisseria flavescens*. Von Lingelsheim's yellow cocci of this genus (*Neis-*

seria flava) and Elser and Huntoon's chromogenic groups (*Neisseria sub-flava*) found earlier than this are separate classes by themselves. They ferment glucose and maltose (Bergey, 1934) though they differ in the type of pigments produced.

The writer was similarly interested in this question in the year 1932 and has studied about 225 epidemic strains during the last two and a half years, all isolated from the cerebrospinal fluid only. Eighty-five strains were found to correspond with the cultural and fermentative characteristics of the meningococcus, whereas 140 strains were found to belong to a different group of Gram-negative diplococci. A preliminary report was published (Sen, 1935a) in which a group of strains was described, which failed to correspond with the meningococcus in cultural and fermentative characteristics.

This group of strains was non-chromogenic, non-hemolytic, grew readily from the primary culture in ordinary nutrient agar, requiring no enrichment fluid in the medium for their growth and *did not ferment any of the sugars*—glucose, maltose, lactose and sucrose, even after 60 generations, as recorded in the preliminary report.

The present report gives a fuller account of the fermentative, biochemical and cultural characteristics of this new group of strains which has been described hereafter as group 6 A, since all the strains isolated with these characteristics have been found to be agglutinated by high titer serum from a particular strain, and also since the type was first isolated from bed No. 6 of a hospital on the first day of cerebrospinal fluid culture.

EXPERIMENTAL

Description of group 6A organisms when first isolated from the cerebrospinal fluid

Gram-negative, biscuit-shaped and flattened diplococci. Grow well on ordinary culture media (plain nutrient agar). Grow well at 22°C. Non-chromogenic. Moist colonies on ordinary and blood agar.

No acid formed in any of the carbohydrate media (glucose, lactose, maltose and sucrose). Do not liquefy gelatin. Agar colonies—small, circular, grayish white with erose margin. Litmus milk—unaltered. Non-hemolytic, non-motile, non-capsulated. Survive for more than

15 days at a temperature of 5°C. Aerobic, facultative. Optimum temperature, 37°C.

Virulence for laboratory animals—very high (killing guinea pigs and rabbits within 24 to 48 hours with symptoms of paralysis of the legs.) Not agglutinated by any of the four Oxford standard typed sera (then in vogue).

During our last study of cerebrospinal fever, we did not obtain *N. flavescens* (Sen, 1935b) (golden-yellow-pigment producing organism), but in the last epidemic we have obtained this chromogenic variety in a small percentage of cases.

That this group of diplococci has many peculiar characteristics is evident. After repeated subculture in ordinary agar for more

TABLE 1

	6A GROUP OF GRAM-NEGATIVE DIPLOCOCCI AFTER 300 GENERATIONS (OLD)	6A GROUP OF GRAM-NEGATIVE DIPLOCOCCI FRESHLY ISOLATED (NEW)	MENINGOCOCCI
Litmus milk	+++	−−−	−−−
Indol reaction	−−−	−−−	−−−
Methyl red	+++	−−−	±±±
Voges-Proskauer	++	−−	−−
Nitrate reaction	++	−−	−−
Ammonia	−−	−−	−−
Methylene blue reductase	++	−−	++
Catalase	−−	±+	++

than 300 generations, it is now found to give positive sugar reactions with glucose and sucrose as well as maltose. Furthermore, the group has changed other biochemical characteristics as well, as is evident from table 1.

From the recent pamphlet issued by the Standard Laboratory (Medical Research Council) along with the standard agglutinating group I and II sera, it appears that "Strains from the cerebrospinal fluid not agglutinable by either serums but otherwise typical, may be considered as group II."

We have tried agglutination tests with recently isolated as well as with 300-generations-old 6 A group of strains against freshly supplied Oxford group sera (two) with negative results. Had

they been otherwise typical, we could have classed them under group II. But they do not conform to the important typical characteristics of the meningococci.

We have studied differences in pathogenicity of freshly isolated "6 A group" meningococci and freshly-isolated typical meningococci, and standard foreign strains of meningococci. The first named is highly neurotoxic and its minimal lethal dose for the mouse is much less than that of the other two. Serum produced in horses against the 6 A group of meningococci, typical locally-isolated meningococci and standard strains, has so far given very good results in the last epidemic in the United Provinces and the Central Provinces of India. A few of our horses, when they were injected with a dose of 6 A group of meningococci more than they could bear, showed stiffness in the muscles on both sides of the spine, to the extent that they could not put their mouths into the feeding tubs easily and walked with difficulty with legs dragging and ultimate paralysis of the legs. In similar conditions with the other strains, we generally observe swelling of the joints only.

This group of strains therefore should be given a separate nomenclature as atypical meningococcus.

SUMMARY

1. A group of Gram-negative diplococci isolated from cerebrospinal fluid of epidemic meningitis cases in 1934–35 was found to differ from the typical meningococcus.

2. This group of strains was found to be capable of growing in ordinary nutrient agar from the very beginning, to have negative fermentation reactions to glucose, maltose and lactose, and to be highly virulent and negative to standard type serum, when freshly isolated and up to 300 generations.

3. After 300 generations, they were found to change their biochemical characters and to show positive fermentation reactions on glucose, maltose, and lactose, though still continuing to be negative to standard group sera.

4. Details of biochemical characters, when freshly isolated and after 300 generations have been tabulated.

I acknowledge with thanks, the assistance rendered to me by my collaborators, Dr. Sudhir Acharyya, Dr. J. Naha, Dr. Phani Dutt, Dr. K. Ganguly and Dr. Satisl Bhattacharya.

REFERENCES

BERGEY, D. H. 1934 Manual of Determinative Bacteriology, p. 71.
BRANHAM, S. E. 1930 Pub. Health Rept., **45**, 845.
BRANHAM, S. E. 1932 Jour. Immunol., **32**, 57.
SEN, B. B. 1935a Journal of Indian Medical Association, April.
SEN, B. B. 1935b Journal of Indian Medical Association, July.

THE INFLUENCE OF OPTICAL ACTIVITY ON THE UTILIZATION OF TRYPTOPHANE FOR GROWTH BY THE DIPHTHERIA BACILLUS[1]

LYLE C. BAUGUESS

Biochemical Laboratory, State University of Iowa, Iowa City

Received for publication May 8, 1936

The use of the optical modifications of tryptophane in metabolism studies of microorganisms has recently received some attention. *Oidium lactis* has been found to utilize *l*- and *dl*-tryptophane equally well when they serve as the sole source of nitrogen in the medium (Bauguess and Berg, 1934). *Escherichia coli*, on the other hand, oxidizes the laevo form readily, but attacks the dextro isomer only slowly, if at all (Woods, 1935).

In the present study is recorded a comparison of the availability of *l*-, *dl*-, and *d*-tryptophane to Yü's strain of the diphtheria bacillus for purposes of growth. It has been known for some time that this organism exhibits scanty growth on a tryptophane-deficient (but otherwise adequate) medium, yet proliferates luxuriantly when small supplements of the naturally-occurring tryptophane are added. No data on the availability of *dl*- or *d*-tryptophane for the purposes of growth of this organism have heretofore been presented.

EXPERIMENTAL

The *l*-, *dl*-, and *d*-tryptophane were prepared in this laboratory. Their physical and chemical constants and the methods used in their preparation are summarized in table 1.

In testing the compounds for growth-promoting ability, they were incorporated in a tryptophane-deficient medium prepared essentially according to the method of Mueller (1935, a). Each liter of the medium consisted of 75 ml. of 10 per cent Liebig's

[1] Included in a paper presented before the American Society of Biological Chemists at Detroit, April, 1935 (Bauguess, L. C., J. Biol. Chem., **109**, v (1935)).

extract, 100.0 mgm. *l*-cystine, 5.00 grams *d*-glutamic acid hydrochloride, 5.00 ml. ethyl alcohol, 25.00 ml. salt mixture,[2] 5.00 grams of the monoamino acid fraction of acid hydrolyzed casein, prepared according to the method of Dakin (1920) and sufficient tenth-normal sodium hydroxide to bring the pH to 7.4.

Into each of 16 vessels were pipetted 125 ml. volumes of this broth. The vessels were then divided into four equal series.

TABLE 1

Methods of preparation and properties of the compounds used in the bacterial metabolism studies

COMPOUND	METHOD OF PREPARATION ESSENTIALLY AS DIRECTED BY	MELTING POINT (UNCORRECTED)	$[\alpha]_D^{20}$ (0.5 PER CENT SOLUTION IN WATER)	NITROGEN	
				Found	Calculated
				per cent	*per cent*
l-Tryptophane.....	Cox and King (1930)	277°	−33.0	13.66	13.72
dl-Tryptophane....	Berg and Potgieter (1931–32)	283°	0.0	13.69	13.72
d-Tryptophane.....	Berg (1933)	281°–282°	+32.5	13.65	13.72

TABLE 2

Growth of the diphtheria bacillus on supplemented tryptophane-deficient media

MILLIGRAMS SUPPLEMENT PER 10 ML.	AVERAGE MILLIGRAM BACTERIAL NITROGEN PRODUCED IN TEN 10 ML. PORTIONS OF A TRYPTOPHANE-DEFICIENT MEDIUM SUPPLEMENTED WITH		
	l-Tryptophane	*dl*-Tryptophane	*d*-Tryptophane
0.000	0.62	0.62	0.62
0.050	1.07	1.05	1.11
0.100	1.39	1.45	1.48
0.500	1.92	1.90	1.96
1.00	2.01	2.09	2.00

One series received no tryptophane supplement. To the second series was added *l*-tryptophane; to the third, *dl*-tryptophane; and to the fourth, *d*-tryptophane. The supplements to the corresponding media of each series were added in amounts of 0.625, 1.35, 6.25, and 12.50 mgm., respectively; the corresponding

[2] The salt mixture used was that previously employed by Mueller (1935, a) and consisted of 70.0 grams NaCl, 2.5 grams K_2HPO_4, 0.15 gram $CaCl_2$, 0.15 gram $MgSO_4$, 0.05 gram $FeCl_3$, 0.5 ml. concentrated HCl, and distilled water to make 350 ml.

media of each supplemented series thus contained 0.050, 0.100, 0.500, and 1.00 mgm. tryptophane per 10 ml., respectively. Ten 10-ml. portions of each medium were then tubed, plugged, and sterilized at 10 pounds pressure for 10 minutes. After sufficient cooling, each tube was inoculated by means of a 3-mm. loop with approximately equal quantities of pellicle from a 24-hour proteose-peptone culture of the strain of diphtheria bacillus first isolated by Yü (1930).[3] After incubation at 37°C. for 60 hours, homogeneity of the bacterial contents of each tube was established and the quantity of bacterial nitrogen produced in each culture was determined by the method of Mueller (1935, b).

The average amounts of bacterial nitrogen produced in each of the media are summarized in table 2. During the experimental period only 0.62 mgm. of bacterial nitrogen was formed in 10 ml. of the unsupplemented medium, whereas these media supplemented with *l*-tryptophane in amounts of 0.050, 0.100, 0.500, and 1.00 mgm. per 10 ml. yielded respectively, 1.07, 1.39, 1.92, and 2.01 mgm. of bacterial nitrogen. These results confirm the findings of Mueller (1935, a). Growth of the organism on the tryptophane-deficient medium supplemented with corresponding amounts of either *dl*- or *d*-tryptophane was essentially identical in each case with the growth occurring in the presence of like amounts of the laevo isomer.

Parallel growth of the Yü strain of the diphtheria bacillus on the media supplemented respectively with the laevo modification, the racemic mixture, and the dextro isomer obviously results from the ability of this organism either to utilize the dextro form directly or to convert it to some readily utilized intermediate common to both *d*- and *l*-tryptophane.

SUMMARY

It is shown that Yü's strain of the diphtheria bacillus is able to utilize *d*-tryptophane as effectively as the naturally occurring isomer for purposes of growth.

[3] I am indebted to Professor J. Howard Mueller of the Department of Bacteriology and Immunology, Harvard University Medical School, for the culture of Yü's strain of the diphtheria bacillus used in this investigation.

REFERENCES

BAUGUESS, L. C., AND BERG, C. P. 1934 Proc. Soc. Exp. Biol. and Med., **31**, 1027.
BERG, C. P. 1933 Jour. Biol. Chem., **100**, 79.
BERG, C. P., AND POTGIETER, M. 1931–32 Jour. Biol. Chem., **94**, 661.
COX, G. J., AND KING, H. T., in CLARKE, H. T. 1930 Organic syntheses, **10**, 100.
DAKIN, H. D. 1920 Jour. Biol. Chem., **44**, 499.
MUELLER, J. H. 1935, a and b Jour. Bacteriol., **29**, 515; **29**, 383.
WOODS, D. D. 1935 Biochem. Jour., **29**, 640.
YU, H. 1930 Jour. Bacteriol., **20**, 107.

THE FERMENTATION REACTIONS OF ERYSIPELOTHRIX RHUSIOPATHIAE

A. W. DEEM AND CHARLES L. WILLIAMS

Department of Bacteriology, The Ohio State University, Columbus

Received for publication May 9, 1936

Owing to the apparently increasing incidence of swine erysipelas in this country, it seemed advisable to study in more detail the cultural characteristics of the causative agent, with a view to simplifying identification and determining cultural differences, if any, in strains of the same organism. The available literature on the fermentation reactions of *Erysipelothrix rhusiopathiae* shows some contradictions.

Marsh (1931) gives the most comprehensive report which we have read on the fermentation reactions of this bacterium, recording that 5 strains tested formed acid in glucose, levulose, galactose, lactose, salicin, dextrin, maltose and glycerol. Edwards (A System of Bacteriology) states that it ferments no sugars except occasionally glucose and lactose. Cornell and Glover (1925) report that, in comparing the bacillus isolated from arthritis in lambs with a strain of the swine erysipelas bacillus of porcine origin, both produced acid in lactose, glucose, maltose and levulose and slight acid in inulin, but no change in sucrose, mannitol, dulcitol, salicin, arabinose and glycerol. In Bergey's Manual of Determinative Bacteriology (1930), the organism is described as fermenting no carbohydrates. Some strains are said by Kelser (1933) to produce a slight acidity in glucose and lactose.

In view of the cultural differences expressed in the literature, it was thought best to obtain for this study as many strains from as widely divergent points as possible. To that end, 37 different strains from the following sources were secured.

4 strains of porcine origin isolated by us from clinical material from widely separated parts of this state.

1 strain from Dr. Hadleigh Marsh of Montana, recovered from a specific arthritis of the lamb.
3 strains from the Pathological Division of the Bureau of Animal Industry of the U. S. Department of Agriculture.
3 strains from Dr. Frank Breed of the Norden Laboratories, Lincoln, Nebraska.
2 strains from the Reichs Gesundheitsamt, Dahlem, Berlin, Germany.[1]
22 strains from the Animal Hygienic Institute, Schleisheim, Munich, Germany.[1]
2 strains from the Animal Hygienic Institute, Mödling, Vienna, Austria.[1]

The swine erysipelas organism grows poorly in ordinary sugar-free (extract) broth. To overcome this difficulty, the following medium was developed and successfully used.

To a quantity of blood corpuscles from citrated swine blood, an equal quantity of tap water was added. This was coagulated in the autoclave at 15 pounds pressure for one-half hour or longer, and was then placed in cheesecloth and the juice pressed out. To this juice was again added an equal quantity of water with enough commercial peptone to make 1 per cent and NaCl to make 0.5 per cent concentration. It was boiled, and filtered through cotton and then through paper. Andrade's indicator was added and the pH adjusted to 7.2. This medium was tubed in small culture tubes and sterilized in the autoclave at 15 pounds pressure for 30 minutes. The broth proved to be sugar-free by biologic test with suitable bacteria. The sugars were sterilized separately in a 20-per-cent solution and added, four or five drops to a tube, aseptically. Each new lot of sugar broth was checked for sterility by incubation. Fermentation of the complex carbohydrates was controlled by test inoculation with suitable bacteria.

When the results with *E. rhusiopathiae* were not conclusive, the tests were repeated, in some instances several times, until definite results were obtained.

Table 1 lists the carbohydrates (and substances usually classed with them) which were used in these tests, together with the

[1] These strains were obtained through the courtesy of Dr. A. F. Schalk of the College of Veterinary Medicine, The Ohio State University.

reactions obtained. This organism freely split the monosaccharides glucose, fructose and galactose, and the disaccharide lactose, but did not metabolize other disaccharides or carbohydrates of larger molecular size. Twenty of the 37 strains fermented mannose, most of them producing only a slight acidity, while the other 17 were negative. Only a few strains produced a slight acidity in arabinose and xylose, with none showing a complete fermentation. In these three sugars there was no correla-

TABLE 1

Fermentation reactions with E. rhusiopathiae

(Results with 37 strains)

CARBOHYDRATES		FERMENTATION REACTION
Monosaccharides		
Hexoses	d glucose	+
	d fructose	+
	d galactose	+
	d mannose	±
Pentoses	arabinose	±
	xylose	±
Disaccharides	lactose	+
	maltose	−
	sucrose	−
Trisaccharide	raffinose	−
Polysaccharides	dextrin	−
	inulin	−
	starch	−
Glucoside	salicin	−
Alcohols	dulcitol	−
	glycerol	−
	mannitol	−

+ = production of acid reaction without gas. − = no acid or gas. ± = variability of reaction among the strains; slight acidity by some strains, others no reaction.

tion between the source of the strains of the organism and their fermentation reactions, both the domestic and the foreign strains showing the same variability.

SUMMARY

Thirty-seven strains of *Erysipelothrix rhusiopathiae* were used to determine the fermentation reactions of this species. Eleven

of these strains originated in the United States and the others were secured from European laboratories.

A special sugar-free broth was prepared in which the organisms were grown readily. Only glucose, fructose, galactose and lactose were fermented by all 37 strains. A slight acid reaction was detected in the cultures of some strains containing mannose, arabinose and xylose. None of the other carbohydrates tested were fermented.

No correlation was apparent between the source of the strains and their fermentation reactions. The uniformity of results demonstrates not only the biochemical activity of this bacterium, but also the reliability of sugar fermentation tests for the identification of this species and its differentiation from organisms of the *Corynebacterium* group with which it may be confused.

REFERENCES

BERGEY, D. H. 1930 Manual of Determinative Bacteriology, 3rd Ed., Baltimore, p. 494.

CORNELL, R. L., AND GLOVER, R. E. 1925 Joint ill in lambs. Vet. Rec., **5**, 833.

EDWARDS, J. T. 1931 Swine erysipelas. A System of Bacteriology, Med. Res. Counc., **8**, 379–389.

KELSER, R. A. 1933 Manual of Veterinary Bacteriology, 2nd Ed., Baltimore, pp. 289–293.

MARSH, HADLEIGH. 1931 The bacillus of swine erysipelas associated with arthritis in lambs. Jour. Amer. Vet. Med. Assn., **78**, (n.s. **31**): 57–63.

THE ESTIMATION OF BACTERIAL POPULATIONS WITH THE AID OF A PHOTOELECTRIC DENSITOMETER

L. G. LONGSWORTH

From the Laboratories of The Rockefeller Institute for Medical Research

Received for publication May 19, 1936

In a study of the amounts of acid formed by cultures of *Lactobacillus acidophilus* when grown at constant pH (Longsworth and MacInnes, 1935, 1936) it was considered desirable to compare the acid production with the bacterial population of the cultures. Two different methods for estimating the latter were employed. These were the determination of the concentration of viable bacteria by the plating method and the measurement of the optical densities of the bacterial suspensions with the aid of a photoelectric densitometer. It is the purpose of this paper (*a*) to describe the densitometer, (*b*) to outline its manipulation and (*c*) to interpret the results in terms of the existing theory of the phenomenon. Comparisons will also be given between the densitometer readings and the plate counts.

The densitometer. The densitometer used in this research utilizes two vacuum phototubes in the bridge circuit shown in figure 1. It is the same in principle but differs widely in constructional details from the instrument described by Stier, Arnold and Stannard (1933–34). A diagram, drawn to scale, of a view of the side of the instrument in cross-section is shown in figure 2.

The phototube P is enclosed and electrically shielded by a light-tight housing constructed from brass tubing. The support for the heat-absorbing water cell at W, the cuvette at C and the diaphragms d is made from square brass tubing. One end of this tubing is soldered to the photoelectric cell housing while the other end passes through a square opening in the side of the lamp house H. A section (aa' in length), of the top side of this tubing is cut

away and fitted with a detachable plate, R, to permit removal of the cuvette and water cell. With this plate in place in the apparatus no extraneous light can enter the phototube. The diaphragms have fixed apertures, $\frac{1}{2}$ inch in diameter, and since no lenses are used the beam of light incident upon the cuvette is a slightly divergent one. The housing for the photoelectric cell is bolted to the base B and additional rigidity for the cell and diaphragm support is furnished by the post A. The right hand

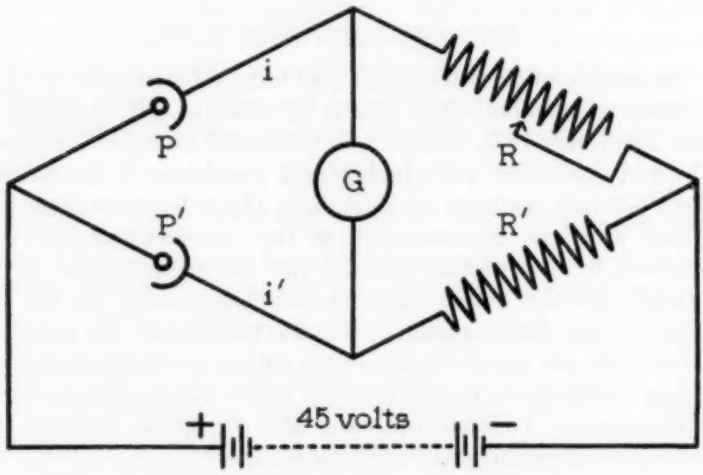


FIG. 1. ELECTRICAL CIRCUIT OF THE DENSITOMETER

P, P', Western Electric 5 A, vacuum photoelectric cells; R', 1000 ohm fixed resistance; R, 10,000 ohm, 4-dial decade resistance; G, galvanometer.

side of the apparatus is the mirror image of the side that has just been described.

The position of the light source L, a 100 w. projection lamp, is maintained with the aid of the upright post D. This post carries a sleeve E to which a horizontal arm of adjustable length is attached, the lamp socket being, in turn, fastened to this arm. The ring collar F, with the set screws G, is attached to the top of the post D, and keeps the upper part of the lamp in place. The entire arrangement is such that the position of the lamp is com-

pletely adjustable[1] and yet, once the proper alignment of the filament with respect to the remainder of the apparatus has been determined, this position may be permanently established.

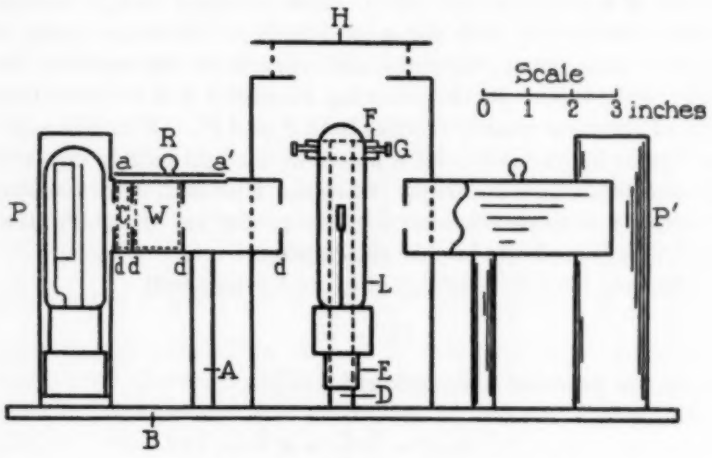


FIG. 2. THE PHOTOELECTRIC DENSITOMETER

Referring again to figure 1, since the current, i, through a phototube is proportional to the light flux, ϕ, reaching it

$$i = k\phi \tag{1}$$

and

$$i' = k'\phi' \tag{2}$$

[1] Any necessary adjustments or replacements have been made in such a manner that the optical density of certain standards which have been adopted retained their initial values. Consequently all measurements made over an extended period of time are strictly comparable. These standards were prepared from a 2-inch square of Corning "snow flake" white glass by cutting it into four equal parts, cementing two of these together with Canada balsam, grinding a third to one-half of its original thickness and leaving the fourth as it was. The thickness of the three standards are 0.981, 2.010 and 4.013 mm. and the densities, referred to a 1-mm. thickness of clear optical glass, are 0.0581_3, 0.1185_7 and 0.2351_5, respectively. In addition to furnishing a check upon the constancy of the densitometer characteristics, such standards as these are also useful in the intercomparison of results made on different instruments.

in which k and k' are "constants" characteristic of the phototubes P and P' respectively. Actually, however, k and k' vary with the temperature and history of the tubes. Moreover, since the response of a photoelectric cell to fluxes of equal energy content varies considerably with the wave length in which the energy is concentrated, these constants also vary with the quality, *i.e.* color, of the light. In the following discussion it is assumed that light of the same quality reaches both P and P'. If turbid material having appreciable color is placed in the light path to one tube this assumption is no longer justified. However, errors arising from this source are eliminated by the proper use of a calibration curve as will be shown later in this paper.

When the phototube bridge of figure 1 is balanced

$$i'R' = iR$$

so that, on reference to equations 1 and 2,

$$R/R' = \frac{k'}{k}\frac{\phi'}{\phi} = K\frac{\phi'}{\phi}$$

Since the fluxes ϕ and ϕ' originate in a common source any variations in the intensity of this source will affect ϕ and ϕ' proportionally and the bridge balance is therefore independent of these variations. Actually, a change of 20 per cent in the line voltage, which corresponds to a 50 per cent variation in the intensity of the light source, produced no change in the reading of the instrument.

In the measurement of the optical density of a suspension the bridge is balanced at $R = R_0$ with the suspending liquid in both cuvettes. This gives the relation

$$R_0/R' = K\frac{\phi'}{\phi_0} \tag{3}$$

A suspension is then substituted for the liquid in the cuvette at C, figure 2, and a new balance at $R = R_i$ obtained, giving the relation

$$R_i/R' = K\frac{\phi'}{\phi_i} \tag{4}$$

The optical density, D, may be defined[2] as the common logarithm of the ratio of ϕ_0 to ϕ_i, *i.e.*

$$D = \log_{10} \frac{\phi_0}{\phi_i} \tag{5}$$

which becomes, on reference to equations 3 and 4,

$$D = \log_{10} \frac{R_i}{R_0}$$

In this derivation it is assumed that the value of K does not change during the interval between the determination of R_0 and R_i. Since k and k' are characteristic of the two quite similar phototubes P and P', it is probable that the ratio, $K = \frac{k'}{k}$, will vary less with time than either k or k' individually. In order to correct for the very slight changes in K which may occur, the author has adopted the procedure of determining R_0 immediately before and after a determination of R_i and using a mean of the two values thus obtained.

The sensitivity of the instrument can best be described with the aid of typical results. With water in both cuvettes a balance was obtained with $R = 1014$ ohms. Changing this resistance by 10 ohms caused a deflection of 5.6 mm. on the scale of a galvanometer with the comparatively low sensitivity of 0.015 microampere per millimeter. Since deflections of 0.2 mm. on the scale of this instrument may be readily detected it is evident that the balancing resistance may be determined to within 0.4 ohm or 0.04 per cent. However, sources of error to be discussed usually render the value of the resistance ratio uncertain by about 0.1 per cent. Consequently the lowest optical density that can be measured with an accuracy of, say, 10 per cent, is $D = \log 1.010 = 0.0043$. As will be shown later this corresponds to a 5 mm. thickness of an *acidophilus* suspension containing 10^7 organisms per milliliter. The measurements reported in this paper were made with the aid

[2] The definition given here is the same as that given by Mestre (1935). Since no reference is made to the thickness of the scattering layer it must be remembered that the optical density, as here defined, will vary with that thickness.

of a cuvette of this thickness and consequently the densities of cell suspensions containing less than 10^7 organisms per milliliter have not been obtained. Preliminary experiments with a cuvette of 5 cm. thickness indicate that very dilute suspensions may be satisfactorily measured by increasing the thickness of the scattering layer.

Manipulation of the suspensions. In the experiments on bacterial growth with automatic pH control, samples were removed at intervals during the development of the culture. One portion of a sample was diluted and used for pouring plates while another portion was used for a determination of optical density. Any changes in the sample that might occur during the interval between these operations would affect the correlation between the plate count and the density. An effort was made to keep these changes at a minimum. When the densities are measured immediately after pouring the plates the time required is only about 10 minutes and any growth occurring in this interval can be neglected.

Changes in the suspension other than growth can also affect the measured optical density and must be eliminated if reproducible values are to be obtained. These changes (assuming no precipitation or other physical changes in the medium) are (*a*) settling of the bacteria and (*b*) their shifting orientation with respect to the incident light. Suspensions of *L. acidophilus* are quite stable and no detectable settling occurs during an actual measurement. When observing suspensions which settle rapidly it should be possible to eliminate errors arising from this source by appropriate agitation of the suspension in the cuvette as will be shown below. The orientation effect, on the other hand, may introduce considerable uncertainty in a determination of density, especially for concentrated suspensions.

Thus, the optical density of a suspension whose particles do not approximate to spherical symmetry depends upon the orientation of these particles with respect to the incident light. Since many bacteria (either individually or in chains or other aggregates), have one dimension greater than the others, the optical density of their suspensions depends upon the orientation of the cells.

It has been the author's experience that the density is a maximum when this orientation is random, a condition realized when the suspending fluid is at rest. If, however, streaming of the fluid occurs the particles partially align themselves with the lines of flow and the measured density is usually decreased. This partial alignment of the bacteria gives to a stirred suspension a characteristic silky appearance.

The effect of agitation of the suspension during a determination has been studied and the results are recorded in table 1. Two types of agitation were tested. Stirring by means of a rotating

TABLE 1

A test of the effect of agitation upon the measured optical density of a suspension of L. acidophilus

TYPE OF AGITATION	OPTICAL DENSITY
No agitation	0.2214
Stirring slowly	0.2125
Stirring rapidly	0.2101
Stirring slowly	0.2130
No agitation	0.2219
No agitation	0.2269
Pumping near edge	0.2130
Pumping in center	0.2269
No agitation	0.2271
Pumping in center	0.2269
Pumping near edge	0.2101

glass rod introduces a uni-directional streaming of the liquid in the cuvette and the resultant alignment of the bacteria causes a decrease in the density as indicated in the table, the effect increasing with the rate of stirring. A second type of agitation was obtained by forcing a few drops of the suspension in and out of a pipet tip with a rapidly oscillating air pressure. When the tip was placed directly over the path of the light through the cuvette an oscillatory motion was imparted to the suspension in the beam and the measured density was the same as with no agitation, indicating an approximate duplication of random orientation. Consequently, agitation of this type may be useful in the measure-

ment of suspensions which settle rapidly. When, however, the tip is displaced from the center a circular, and hence uni-directional, flow of liquid is established in the beam and the density decreases as indicated in table 1. The data of this table also indicate that the orientation characteristic of a given type of agitation is reproducible. These effects, however, are relatively more pronounced in concentrated suspensions than in dilute ones and may introduce errors in observations of the former because of the inability of the operator to reproduce a given orientation. Since thermal gradients in the instrument and the heating effect of the light beam may set up convection currents in the suspension it is probable that a random orientation of the particles is never completely established.

Most of the values reported in this paper were obtained without mechanical agitation. It has been observed that during the first few seconds after the introduction of the suspension into the cuvette, the density rises slightly to a flat maximum after which it decreases slowly. The establishing of random orientation, as the agitation which arises from the filling of the cuvette subsides, accounts for the initial rise in the density. The subsequent decline is probably due to the feeble convection currents set up in the suspension by thermal gradients in the apparatus. The maximum value has been taken as the correct one since the orientation is approximately random during this interval.

Densitometry. In a determination of optical density it is necessary to measure (*a*) the light flux, ϕ_i, which reaches the phototube after escaping from the suspension, and (*b*) the flux, ϕ_0, which is collected when this suspension is replaced by an equal thickness of the suspending medium. When a beam of light impinges upon a turbid medium a part, ϕ_u, of the light is transmitted without deviation, another part, ϕ_a, is absorbed and the remainder, ϕ_s, is scattered in all directions by reflection and refraction. In any real instrument used for the measurement of optical density the collected flux, ϕ_i, consists of ϕ_u and that portion of ϕ_s included within a solid angle, Ω, which is determined by the design of the instrument. The relation between these quantities is

$$\phi_i = \phi_u + \int_0^{\Omega} \phi_s d\Omega \tag{6}$$

Since the value of Ω varies, in general, from one instrument to another the density defined by equation 5 is a function of the instrument used in making the determination. Moreover, a comparison of equations 5 and 6 shows that the density of a given suspension is a maximum when measured on an instrument which excludes the scattered light.

The relation of the measured density to the concentration and thickness of the scattering layer is, in general, a complicated function of these variables. Both theory (Ryde 1931) and experiment (Dreosti 1931, Mestre 1935) indicate, however, that a density, $\bar{D}$, defined by the relation

$$\bar{D} = \log \frac{\phi_0}{\phi_u} \tag{7}$$

(*a*) is proportional to the thickness of the scattering layer for a given suspension, and (*b*) is proportional to the concentration for a given thickness (Beer's law applied to turbid media) for large variations in these variables. The term concentration, as used here and elsewhere in this paper, refers only to the factor by which a stock suspension is diluted, since in this manner of altering the concentration only the number of scattering centers per unit volume is changed and not their nature.

If an instrument is designed so that the incident light is a carefully collimated beam from a small source which, after passing through the turbid medium, is brought to focus on a correspondingly small aperture in front of the collector, most of the light which reaches the latter will have been transmitted without deviation. Dreosti (1931) and Mestre (1935) have shown that these highly selective instruments obey Beer's law over a large range of density. Unless carefully corrected lenses of large aperture are used, a selective instrument has the limitation, however, that the quantity of light reaching the collector is small and if the latter is a phototube, amplification of the photoelectric current is necessary if the desired sensitivity is to be attained.

Amplification and the use of lenses may be avoided if the collected light includes some of that which is scattered in the forward direction since the light source may then have a relatively large area of high intrinsic brilliance. The instrument used in the present research is only partially selective. Since similar instruments have been used in other laboratories (Stier, Arnold and Stannard, 1933–34; Richards and Jahn, 1933; Sanford, Sheard and Osterberg, 1933) and are available commercially[3] it is desir-

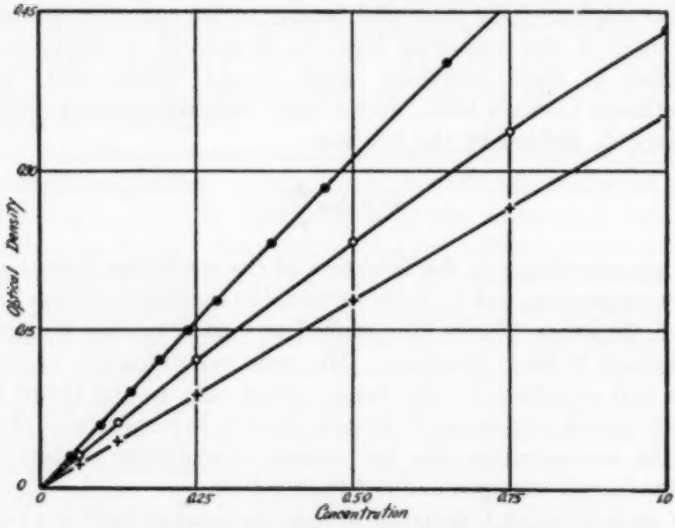


FIG. 3. CALIBRATION OF THE DENSITOMETER WITH SUSPENSIONS OF INDIA INK ●, LACTOBACILLUS ACIDOPHILUS ○, AND YEAST +

able to indicate their limitations and show how these may be largely overcome.

The behavior of the instrument can best be discussed with some concrete examples. In figure 3 are plotted the results of optical density measurements on serial dilutions of stock suspensions of India ink, yeast and *L. acidophilus*. The relation between the

[3] The A. P. C. Turbidity Meter of Eimer and Amend and the Photelometer of the Central Scientific Company are examples. The Suspensiometer described by Gates (1920) and sold by the Empire Laboratory Supply Company is also widely used by bacteriologists.

optical density and the concentration is represented by curves which are very nearly straight lines. The data used in the preparation of the figure are given in table 2. Each of the series of measurements required less than an hour for its determination. At the conclusion of the measurements the densities of some of the suspensions first observed were again measured and in all cases

TABLE 2

Calibration of the densitometer with various suspensions

L. ACIDOPHILUS*		YEAST†		INDIA INK‡	
Conc.	$D_{(obsd.)}$	Conc.	$D_{(obsd.)}$	Conc.	$D_{(obsd.)}$
0.0078	0.0040	0.0078	0.0030	0.049	0.030
0.0156	0.0080	0.0156	0.0060	0.097	0.059
0.0312	0.0165	0.0312	0.0111	0.145	0.091_5
0.0625	0.0310	0.0625	0.0220	0.192	0.121
0.125	0.0620	0.125	0.0445	0.236_5	0.149
0.25	0.1212	0.25	0.0885	0.370_5	0.231
0.50	0.2330	0.50	0.1775	0.454_5	0.284
0.75	0.3369	0.75	0.2650	0.650	0.402
1.00	0.4325	1.00	0.3518	1.000	0.602
α.....	0.505		0.358		0.635
β^2.....	0.0725		0.0064		0.027
Average deviation.....	0.9%		1.9%		1.1%
ρ.....	0.53		0.22		0.26

* Twenty-four-hour culture separated from medium, washed and resuspended in water.

† Twenty-four-hour culture of *Saccharomyces cerevisiae* Hansen (A. T. C. S. No. 4360) in Williams' medium, separated from medium, washed and resuspended in 2 per cent sugar solution.

‡ Ammoniacal suspension.

checked the earlier readings satisfactorily, indicating that no significant changes in the suspensions had occurred.

The relation between the optical density D and the concentration C for the three series of measurements mentioned above can be represented accurately by the equation

$$D = \alpha C - \beta^2 C^2 \tag{8}$$

The values of α and β^2, as determined from the slope and intercept of a plot of $\frac{D}{C}$ *versus* C, are given in table 2. This table also contains the average deviation of the values computed with the aid of equation 8 from the observed densities.

Although the numerical values of α and β^2 recorded in table 2 depend upon the concentration scale, which is entirely arbitrary since the concentration of each stock suspension was taken as unity, the value of the ratio

$$\frac{\beta}{\alpha} = \rho \tag{9}$$

is *independent* of this scale. If there were a strict proportionality between the optical density and the concentration, β, and hence ρ, would be zero. Consequently the deviation of ρ from zero is a measure of the lack of proportionality between the density and the concentration.

Microscopic examination of suspensions of *L. acidophilus* indicates that the individual cells are optically empty, thus differing markedly in this respect from yeast. Consequently, as von Angerer (1924) and Mestre (1935) have emphasized, the cells of such suspensions might be expected to act as tiny lenses of long focal length and thus to scatter the light mainly in the forward direction with only slight deviation from the direction of incidence. Thus, the contribution of the scattered flux, $\int_0^\Omega \phi_s d\Omega$ of equation 6, to ϕ_i would be large as compared with that for suspensions in which the scattering is more nearly isotropic. Since it is assumed that the scattered flux does not obey Beer's law, suspensions for which $\int_0^\Omega \phi_s d\Omega$ is an appreciable portion of ϕ_i might be expected to exhibit a relatively large value of ρ. This is observed in the case of *L. acidophilus* suspensions.

Although a suspension may appear white to the eye, its optical density may vary with the color of the incident light and this is another factor which may affect the value of ρ. Thus a water suspension of *L. acidophilus*, which had a density of 0.63 as

measured on a Zeiss Pulfrich photometer with an S43 (red) filter, had a density of only 0.25 with the S72 (blue) filter.[4] The density of a suspension of yeast, on the other hand, was independent of the wave length of (visible) light used in its determination. The influence of color upon the value of ρ has not yet been determined.

Since the measured densities of suspensions of *L. acidophilus* are not strictly proportional to the concentration it is desirable to make a small correction for this lack of proportionality. This may be done most conveniently with the aid of equation 8. If the corrected values are indicated by D^*, then

$$D^* = \alpha C \tag{10}$$

Elimination of C between equations 8 and 10 gives, on reference to equation 9, the relation

$$D = D^* - \rho^2 D^{*2} \tag{11}$$

in which D^* is a function only of D and ρ, and hence independent of any concentration scale. Although deviations of ρ from zero are due partly to the incomplete selectivity of the densitometer, it has also been shown from the data given in table 2 that variations in this quantity are also due to differences in the optical properties of the suspensions. Before equation 11 can be used to correct the observed densities of *L. acidophilus* cultures it must be shown that the value of ρ does not depend upon the age of the culture used in the preparation of the stock suspension described in table 2. This was done by diluting threefold a portion of each sample taken from an *L. acidophilus* culture at various stages in its development and measuring the densities of both the diluted and the original sample. Values of ρ were computed for each pair of measurements and deviated but slightly and in a random manner from a mean value of 0.48. A value of $\rho = 0.5$ has been used in correcting all measurements on *L. acidophilus* cultures with the aid of equation 11. A convenient method of using this calibration formula is as follows. Values of D are computed for different values of D^* and a plot of $D^* - D$ as ordinate against D

[4] For a discussion of the color of "white" suspensions, see Teorell (1930).

as abscissa is prepared. The value of an ordinate corresponding to a measured density is then merely added to the measured value to give the corrected density, D^*.

COMPARISON OF MEASUREMENTS OF OPTICAL DENSITY WITH PLATE COUNTS

With the aid of the densitometer, the construction and manipulation of which have been described in the first part of this paper, optical densities have been measured at intervals during the development of *L. acidophilus* cultures. These studies have been made for cultures grown under a variety of experimental conditions, the pH being maintained at a constant value in most of the experiments.

Cultures grown with pH control. The results of an experiment on the growth of *L. acidophilus* at a constant pH of 6.0 are recorded in table 3. Inoculation was made at zero time and samples were then removed for study as indicated in the first column of the table. The observed densities, D, are recorded in the second column. These values are subject to two corrections. The first one reduces the observed density to the value it would have if there were a strict proportionality between density and concentration. This correction has been discussed in the preceding section and the corrected values, D^*, are recorded in the third column of table 3. A second correction is necessary when results obtained in experiments performed under different conditions are to be compared and arises from the diluting effect of the alkali which is added in maintaining the culture at a given pH. This correction has been adequately discussed elsewhere (Longsworth and MacInnes, 1936). It should be emphasized, however, that any correlation between plate count and optical density for a given experiment is independent of the second correction, since both sets of measurements are multiplied by the same factor. The fully corrected densities, $D^{*\prime}$, are given in the fourth column of the table and have been plotted as the open circles in figure 4.

The corrected plate counts, C', are recorded in the fifth column of the table and are indicated by the crosses in figure 4. Each

figure in this column is the average from four plates. The mean deviations of individual plates from these figures varied from 1.8 to 10.1 per cent with an average deviation of 5.0 per cent.

TABLE 3

A comparison of measurements of optical density with plate counts during the growth of L. acidophilus with automatic pH control

Temperature, 38°. pH, 6.0. Medium: 2 per cent neopeptone, 8 per cent galactose. Gas: CO_2 2.6 per cent, N_2 97.4 per cent. Titrating fluid: 1.94 N NaOH.

TIME	OPTICAL DENSITY			PLATE COUNT, $C' \times 10^6$ PER ML.	$\frac{C'}{D^{*'}} \times 10^9$
	D	D^*	$D^{*'}$		
hours*					
8.0	0.0029	0.0029	0.0029	5.09	1.76
10.0	0.0064	0.0064	0.0064	14.2	2.22
12.0	0.0142	0.0142	0.0142	34.9	2.46
14.0	0.0288	0.0290	0.0291	52.4	1.80
16.0	0.0507	0.0515	0.0518	129	2.49
18.0	0.0837	0.0857	0.0866	291	3.36
20.0	0.1230	0.1272	0.1295	467	3.61
22.0	0.1702	0.1782	0.1830	650	3.55
24.0	0.2203	0.2340	0.2427	821	3.38
26.0	0.2777	0.3003	0.3156	1,074	3.40
28.0	0.3260	0.3582	0.3829	1,234	3.22
30.0	0.3948	0.4440	0.4831	1,647	3.41
33.0	0.4610	0.5310	0.5984	1,956	3.27
48.0	0.4967	0.5811	0.7084	2,832	4.00
55.0	0.5044	0.5919	0.7375	2,727	3.70
72.0	0.4638	0.5349	0.6890	2,626	3.81
83.0	0.4677	0.5403	0.7040	1,388	1.97
100.0	0.4367	0.4989	0.6551	386	0.59

* Plate counts at 0.0, 2.0, 4.0 and 6.0 hours were 0.230, 0.354, 0.757 and 1.80 $\times$ 10^6 per milliliter, respectively.

The last column of table 3 contains the value of the ratio

$$R = \frac{C'}{D^{*'}}$$

as computed from the data of the two preceding columns. If the density were proportional to the plate count at every stage in the development of the culture this ratio would be a constant. In view of the marked morphological changes (see, for example,

Bayne-Jones and Adolph (1932–33)) that are known to occur in many bacterial cultures a constant ratio is hardly to be expected. Actually, however, the *value of R in table 3 is very nearly constant throughout a period in which more than 90 per cent of the total growth occurs.* Thus from the 18th to the 72nd hour the average deviation of this ratio from a mean value of 3.52×10^9 is only 5.5 per cent. This is shown graphically in figure 4 in which one smooth curve represents both densities and plate counts almost equally

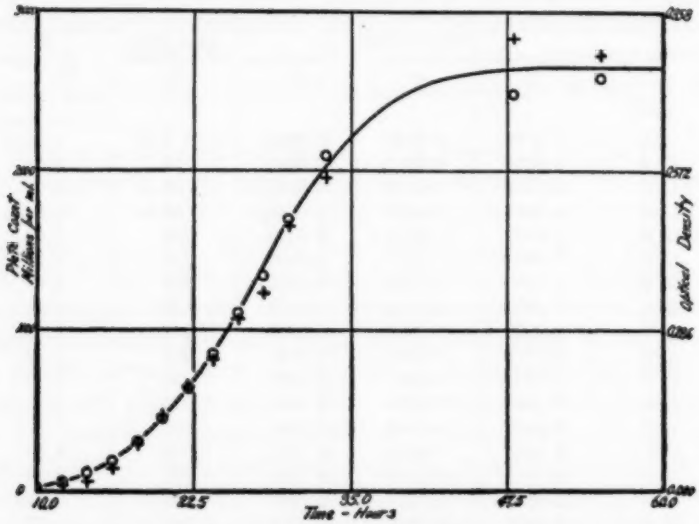


FIG. 4. THE VARIATION, WITH TIME, OF THE PLATE COUNT AND THE OPTICAL DENSITY OF A CULTURE OF L. ACIDOPHILUS GROWN AT A CONSTANT pH OF 6.0

well. Moreover, the deviations from constancy exhibited by this ratio in the young culture, and again in the old culture, are in qualitative accord with the physiological changes occurring during these periods.

If the *logarithm* of the plate count is plotted as ordinate against the time as abscissa a curve (not shown) is obtained which exhibits the usual features of a normal growth cycle. After a short initial period (1) of increasing growth rate the culture passes into

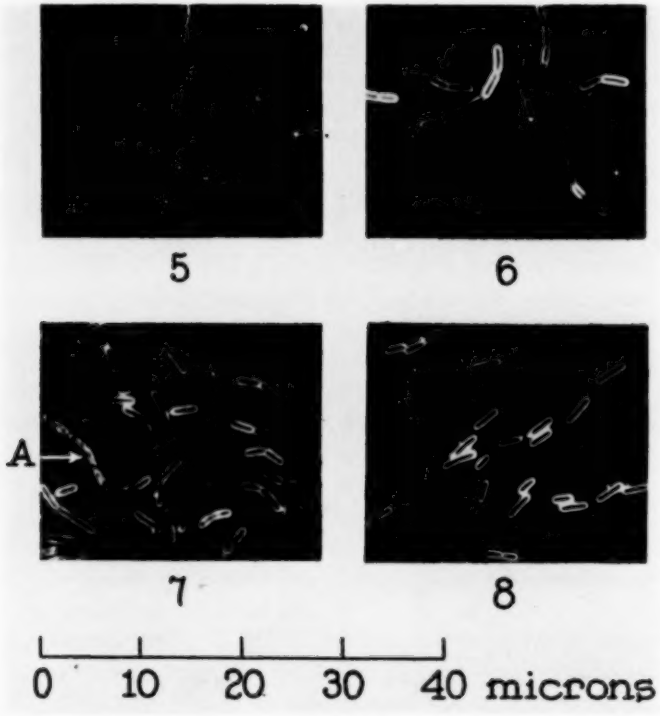
(2) its "logarithmic" phase and remains in this phase until about the 14th hour after which (3) the growth rate slowly decreases until (4) a stationary population is attained at about the 40th hour. At about the 75th hour (5) the plate count begins to decrease. Over 90 per cent of the total growth occurs in the third period and it is during this and the succeeding interval that the ratio of the plate count to the optical density is constant.

With *L. acidophilus* and an inoculum of the size used in our experiments it has been impossible to obtain accurate density measurements during either the first phase or the first part of the second phase of growth. By the time the culture has developed a sufficient optical density for a precise determination of this quantity it is in the later part of the logarithmic phase. The initial values of R are relatively low, and increase, during the transition of the culture from the second to the third phase, to the constant value characteristic of the latter phase. During the fifth phase the plate count decreases rapidly whereas the density changes very little with the result that R decreases.

An interpretation of the observed changes in R has been derived chiefly from microscopic examination of the culture in its various phases. The most significant changes occurring in the morphology of the bacteria are illustrated in figures 5, 6 and 7 which are reproductions of photomicrographs of the living, unstained cells with dark field illumination. These changes may be summarized as follows. The bacteria from a culture in its logarithmic phase (fig. 5) are sausage-shaped cells linked together in chains of several units each. Each cell in the chain is optically empty, the apparent thickness of the cell wall decreasing as the resolving power of the microscope is increased. During this phase of growth the ratio of the plate count to the optical density has the low value of about 2×10^9.

Later in the development of the culture the following morphological changes are observed. Separation of the chains shown in figure 5 into groups containing fewer cells, as shown in figure 7, results in an increase in the plate count without a corresponding increase in the density. This may account for the observed increase in the value of R to 3.5×10^9 during the transition of the

culture from its "log" phase to that in which the growth rate is decreasing. In this explanation of the observed change in R it is assumed that the process of pouring the plates is without effect upon the average number of cells per chain. Insofar as agitation of the suspension during this operation affects the number of cells



FIGS. 5-8. PHOTOMICROGRAPHS OF L. ACIDOPHILUS IN DIFFERENT STAGES OF GROWTH

per chain this assumption is certainly justified since this agitation is not as severe as that to which the entire culture is continuously subjected. It is also assumed that a chain of cells produces only one colony.

A change which tends to increase the optical density of the

suspension without a corresponding increase in the plate count is illustrated by some of the cells in figure 6 and consists of a uniform increase in the apparent thickness of the cell wall. This is often followed by a non-uniform extension of the thickening to the interior of the cell until the typical appearance of the granulated cell, *A*, shown in figure 7, is attained. The debris which is prevalent in an old culture has an appearance which suggests that the particles arise from the disintegration of granulated cells. The constancy of the plate count-optical density ratio during the third and fourth phases of growth may be due, in part, to a compensation between these changes which tend to increase the density and that described in the preceding paragraph which tends to increase the plate count.

A very important factor about which, unfortunately, little can be said, is the contribution of non-viable cells and bacterial debris to the optical density. No attempt has been made to correlate optical changes in the cell with loss of viability. The constancy of R during a large portion of the growth cycle does not exclude the possibility that a proportion of the cells have lost their ability to form colonies. It is only during the final phase of growth, in which the plate count decreases quite rapidly as compared with the density, that the contribution of non-viable cells to the latter becomes obvious.

The trends of the ratio of the plate count to the optical density in the experiment just described are representative of all cultures grown with pH control. The numerical value of R, taken during the phases of growth in which it is constant, varies with the conditions of the experiment as is illustrated by the data of table 4. In this table the composition of the media and gas mixtures used in different experiments are recorded in the first five columns. The bacterial population of the culture when this is stationary and at its maximum are recorded in the sixth column. It is during this and the preceding phase of growth that the constant values of R given in the last column of table 4 were obtained. These values, while nearly independent of alterations in the media and gas, exhibit variations that may have interesting significance. Thus the value of R is consistently high when the sugar content of the

medium is high. Similarly the value of R was increased somewhat when the carbon dioxide content of the gas mixture was

TABLE 4

The variation of the ratio of the plate count to the optical density with changes in the medium and gas for cultures of L. acidophilus grown at a constant pH of 6.0

MEDIUM		GAS			PLATE COUNT (STATIONARY), $C \times 10^6$ PER ML.	$R \times 10^3$
Protein	Sugar	CO_2	O_2	N_2		
		per cent	*per cent*	*per cent*		
.2% peptonized milk	3% galactose	7.6	2.1	90.3	380	2.8
2% peptonized milk	3% galactose	8.0	1.9	90.1	800	2.8
2% peptonized milk	3% galactose	8.2	<0.2	91.6	600	2.8
2% peptonized milk	3% galactose	41.6	<0.2	58.2	700	3.2
2% peptonized milk	8% galactose	8.2	<0.2	91.6	1,200	3.6
2% neopeptone	8% galactose	2.6	0.0	97.4	2,800	3.5
2% neopeptone	8% glucose	3.8	0.0	96.2	2,600	3.6
2% neopeptone	8% glucose	2.4	2.4	95.2	1,200	3.4

TABLE 5

A comparison of optical density measurements with plate counts during the growth of L. acidophilus without pH control

Temperature, 38°. Medium: 2 per cent neopeptone, 8 per cent glucose

TIME	pH	OPTICAL DENSITY, D^*	PLATE COUNT, $C \times 10^6$	$R = C/D^* \times 10^3$
hours				
10.0	6.47	0.0028	7.04	2.51
12.0	6.38	0.0060	11.0	1.83
14.0	6.25	0.0099	25.1	2.54
16.0	6.00	0.0172	36.8	2.14
18.0	5.59	0.0279	66.6	2.39
20.0	5.10	0.0425	123	2.89
22.0	4.71	0.0599	224	3.74
24.0	4.44	0.0805	281	3.49
26.0	4.26	0.1016	439	4.32
30.5	4.03	0.1310	667	5.09
34.0	3.93	0.1450	708	4.88
47.0	3.79	0.1761	780	4.43
54.0	3.72	0.1750	893	5.10
73.5	3.63	0.1865	867	4.65
95.0	3.54	0.1960	843	4.30

increased from 8 to 40 per cent, an effect that may throw light upon the rôle of this gas in bacterial respiration. Changes in the

oxygen content of the gas mixture were without effect upon the value of R.

Cultures grown without pH control. A variable which has a more pronounced effect upon the plate count-optical density ratios than any of those discussed in connection with table 4 is the pH. When a culture of *L. acidophilus* was grown in the usual manner without pH control the results shown in table 5 were obtained. The initial variation of R with the time is quite similar to that of table 3 but, as the pH of the culture decreases, this ratio increases to a much higher value than in the experiment with pH control. The low pH of the medium apparently favors a separation of the chains of cells into their individual units as illustrated in figure 8, which was obtained from a culture at pH 3.8. This separation increases the plate count without a corresponding change in the optical density and accounts adequately for the observed trend of the values of R.

CONCLUSION

The results of this part of our work indicate that, throughout the development of a culture of *L. acidophilus* until the viable count begins to decrease, the ratio, R, of the plate count to the optical density varies by a factor of not more than about 2 even when the bacteria are grown under widely different conditions. The observed changes in R can be greatly reduced by the control of experimental variables, of which an important one is the pH. Once the relation between the plate count and the density has been established for a given set of experimental conditions the ease and precision with which measurements of the latter can be made recommend this method for the estimation of bacterial populations.

It is a pleasure to acknowledge my indebtedness to Dr. D. A. MacInnes of these Laboratories for his helpful suggestions during the course of the work. The author is also indebted to Dr. Harold Mestre for stimulating and clarifying conversations on the subject of densitometry.

SUMMARY

A photoelectric densitometer has been described with which measurements of optical density may be quickly and accurately made. The necessary precautions in the use of the instrument for the determination of the densities of bacterial suspensions have been outlined. The densities of suspensions of *Lactobacillus acidophilus* have been determined at intervals during the growth period. The results of such measurements are available almost immediately, a great advantage over the plate count method. If the pH of the culture is held constant these measurements are proportional to the plate counts over an interval in which more than 90 per cent of the total growth occurs. The variations of the proportionality factor with changes in the medium and with the pH have also been studied.

REFERENCES

ANGERER, K. v. 1924 Arch. Hyg., **93**, 14.

BAYNE-JONES, S., AND ADOLPH, E. F. 1932–33 Jour. Cell. and Comp. Physiol., **2**, 329.

DREOSTI, G. M. 1931 Phil. Mag., **11**, 801.

GATES, F. L. 1920 Jour. Exp. Med., **31**, 105.

LONGSWORTH, L. G., AND MACINNES, D. A. 1935 Jour. Bact., **29**, 595.

LONGSWORTH, L. G., AND MACINNES, D. A. 1936 Jour. Bact., **31**, 287.

MESTRE, H. 1935 Jour. Bact., **30**, 335.

RICHARDS, O. W., AND JAHN, T. L. 1933 Jour. Bact., **26**, 385.

RYDE, J. W. 1931 Proc. Roy. Soc. London, Ser. A, **131**, 451.

SANFORD, A. H., SHEARD, C., AND OSTERBERG, A. E. 1933 Amer. Jour. Clin. Path., **3**, 405.

STIER, T. J. B., ARNOLD, W., AND STANNARD, J. N. 1933–34 Jour. Gen. Physiol., **17**, 383.

TEORELL, T. 1930 Kolloid-Z., **53**, 322.

A STUDY OF THE CHARACTERISTICS OF VARIANTS DERIVED FROM SINGLE CELLS OF ESCHERICHIA COLI

JOHN C. TORREY AND ELIZABETH MONTU

Department of Public Health and Preventive Medicine, Cornell University Medical College, New York City

Received for publication May 22, 1936

A number of studies on *Escherichia coli* dissociation have appeared since Arkwright (1921) introduced the terms S (smooth) and R (rough) for variants in the typhoid-colon-dysentery group and established some of their distinguishing properties. These studies on *E. coli*, Gratia (1921), Bergstand (1923), Dulaney (1928), Nungester and Anderson (1931), O'Neal (1933), Rennebaum (1933), have not been based, with the possible exception of that of Gratia, on a single cell strain obtained under controlled microscopical conditions. There would seem to be certain clear advantages in basing an investigation of this character on single cell lineage dissociates, particularly in that only thus may the limits of potential variability inherent in the protoplasm of a single normal S organism be determined. In this communication there are reported morphological, biochemical and virulence changes associated with each of the several dissociative phases of an unusually virulent *Escherichia coli* (hemolyticus) strain under single cell controlled conditions, together with a discussion of the antigenic structure of these dissociates based on agglutination experiments. In another paper immunological findings will be described.[1]

SOURCE OF STRAINS

These hemolytic *E. coli* strains were isolated some years ago (December, 1931) from the purulent intestinal discharge of an

[1] A preliminary report on the immunological study appeared in the Journal of Immunology, 1935, **29**, 64.

acute, febrile case of non-specific ulcerative colitis in an adult female. An active and strain specific bacteriophage was recovered from the stool and later employed in this dissociation study. The patient's serum agglutinated these hemolytic strains in dilutions from 1:40 to 1:160, but not the associated non-hemolytic *E. coli*. When first isolated 0.0025 cc. of a 24-hour plain broth culture was lethal in 24 hours when inoculated intraperitoneally in white mice, and 0.5 cc. intravenously for a rabbit.

SINGLE CELL ISOLATIONS AND METHODS OF DISSOCIATION

Two original *E. coli* isolations have been studied from the dissociative standpoint. One, strain 3, formed a smooth domed colony with occasionally a raised rim of mucoid material or "schleim-wall," often marked with fine radial striae, when incubated at 37°C. 24 hours, and then for the same period at room temperature (23°) (fig. 2). Various members of the *Salmonella* group and also *Bacillus abortus equi* have been described as developing this type of colony under these temperature conditions (System of Bacteriology, 1929).

In the primary single cell isolation a typical S colony (strain 3) was seeded into plain broth, incubated three hours and then single cells were isolated with the Chamber's micro-manipulator according to the Kahn (1922) technic and each seeded into a tube of plain broth (pH 7.2). Acknowledgment for assistance in this part of the work is made to my associate, Dr. Morton C. Kahn. From single cell growth thus obtained, dissociates were secured by various measures and these in turn isolated in single cell culture.

In labelling these dissociates, the first two early R types have been designated in accordance with Hadley's (1927) classification O_1 and O_2 as they are intermediate between normal S and the frankly R forms. These dissociates with two definitely R types have been carried in semi-solid hormone agar and stored in the refrigerator (8°C.) with frequent checks on stability.

Dissociates 3-O_1, and 3-O_2 developed during the growth of 3-S in plain or glucose broth (pH 7.2); 3-R_2 was obtained by growing 3-O_2 in 10 per cent immune S serum in plain broth. The 7-R_1

dissociate was also obtained by growth in immune serum broth. The 3-O_1 and 3-O_2 dissociates may revert to the S phase during growth in plain broth, but 3-R_2 is apparently stabilized in the extreme rough phase as various measures such as growth in S and also R immune serum broth, passage through white mice, inoculation of a mixture of the R_2 cells and sterile S broth culture filtrate intraperitoneally have been unsuccessful in effecting any change toward S. As will be shown, the 3-O_2 and 7-O_2 are typical for R as regards loss of virulence, sedimenting growth in plain broth culture, flocculation in normal saline and to some degree in colony morphology, but are S in antigenic constitution. The 7-R_1 dissociate is so labelled because unlike 3-R_2 it has occasionally reverted toward the S phase as a result of passage through mice. The 7-R_1 (mucoid) is a dissociate from 7-R_1, after the latter had been passed through R immune serum broth for 8 generations. During this process it regained a capsule and acquired the antigenic constitution of the O phase.

DIFFERENCES IN DISSOCIATIVE POTENTIAL OF SINGLE S AND O ORGANISMS

In bacterial dissociation studies the relative degree of segregation of R dissociative potentialities in a single organism from a colony supposedly pure for normal S has apparently attracted little attention. There has been a tendency to assume that the individual members of a normal S colony are much alike, if not identical. For such an analysis it is, of course, essential to start each culture from a single organism. In table 1 enough examples are given to demonstrate that after repeated selective colony fishing and plating the component bacilli of a normal S colony of *E. coli* varied in their potential early R content and to still greater extent was dissimilarity apparent for individual bacilli of an O or intermediate colony. On the other hand, single bacilli from the frankly R colonies remained stable for that type.

In these experiments a single bacillus was planted into a tube of plain broth (pH 7.2), incubated at 36°C. and surface platings were made on purple lactose agar at the indicated growth period intervals with a later differential colony type count. The rela-

tive numbers of S and O colonies are expressed in percentage of the total colony count (200–300). Of three cells from a normal S colony only one was found to be pure, or nearly so, for S; the other two revealed a dissociation potential of from 28 to 76 per cent at the end of a three to four weeks growth in the broth medium. The five single organisms of the O_2 (early R) stage showed even more marked differences in stability. After 5 to 8 days incubation, the percentage of reversion to S ranged from 2.7

TABLE 1

Single cell isolations

Single cells from 3-hour plain broth cultures grown in plain broth (pH 7.2) and incubated at 36°C. Plated at indicated intervals. Colony type incidence expressed in per cent.

E. COLI DISSOCIATE TYPE		DATE	1 TO 3 DAYS		5 TO 8 DAYS		12 TO 15 DAYS		23 TO 30 DAYS		80 DAYS	
			S	O_2	S	O_2	S	O_2	S	O_2	S	O_2
S (strain 3)	a..	11/10/32	100.0	0.0					72.0	28.0		
	b..		100.0	0.0					99.0	1.0		
	c..	*11/ 8/33	99.0	1.0	97.5	2.5	59.5	40.5	24.0	76.0	26.0	74.0
O_2 (strain 3)	a..		0.2	99.8	3.4	96.6	8.7	91.3	0.2	99.8		
	b..	1/17/33	0.5	99.5	3.9	96.1	1.6	98.4	0.0	100.0		
	c..		0.0	100.0	2.7	97.3	0.0	100.0	0.0	100.0		
	d..	2/19/34	0.0	100.0	12.0	88.0	0.0	100.0	30.0	70.0		
	e..		2.5	97.5	43.5	56.5	41.0	59.0	42.5	57.5		
			S	R	S	R	S	R	S	R		
R_2 (strain 3).....		1/21/34	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0		
R_1 (strain 7).....		11/ 4/32	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0

* 3-S, single cell culture (*b*) of November 10, 1932, re-isolated in single cell.

to 43.5; after 23 to 30 days in three instances these S elements had apparently again dissociated to the early R phase while in the other two cultures the S element now amounted to 30 and 42.5 per cent respectively. The swing back and forth between S and early R in one culture (d) is clearly marked and appears of a cyclic type. Dissociation to the frankly R phase did not occur under these conditions.

Complete stabilization in the S phase was obtained in the fol-

lowing way: A single organism from an S colony was seeded into a tube of plain broth (pH 7.2) and incubated at 35° for 43 days. On plating 36 per cent of the colonies were S in type and 64 per cent early R. An S colony was seeded into a broth tube and incubated 28 days. On plating 99.5 per cent of the colonies were S and 0.5 per cent early R. Five of these S colonies were seeded into broth and incubated 35 days. On the final plating three tubes yielded 100 per cent S colonies. These were proved to be stabilized in the S phase by prolonged aging in broth medium and by growth in broth plus immune S serum for 9 generations. No decrease in virulence was found on completion of this stabilizing process.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

The colony and organism morphology and certain biochemical characteristics of the normal S and gradient O and R dissociates of this *E. coli* strain are summarized in table 2 and illustrated in figures 1 to 15. The intermediate O stages in the progression to extreme "roughness" frequently give rise to minute slowly growing colonies when seeded on purple lactose agar plates and incubated 24 hours at 36°, as illustrated in figure 3. After further incubation these "G" like colonies (but not the "G" of Hadley as regards morphology and filterability) attain the normal size. Mucoid dissociates, of the type shown in figure 4, have been obtained from the normal 3-S, 3-O_2 and 7-R_1, but not from 7-S and 3-R_2. They have arisen at times spontaneously during the course of growth in plain broth and frequently in cultures in semi-solid hormone agar after several months at refrigerator temperature (5°C.). Thus strain 7-R_1, in a non-mucoid stage, after six months storage in the refrigerator yielded nearly 100 per cent mucoid colonies when plated after 7 days growth in plain broth; also 3-S and 3-O_2 have shown such colonies after prolonged refrigeration and without preliminary growth in broth. They may also be obtained by growth in immune (R) serum broth and by growing on phage treated agar plates. These mucoid colonies may become very large on purple lactose agar after several days growth at room temperature, ranging in diameter up to 1.7 cm. for 7-R_1.

The mucoid state is apparently not due to the formation of enlarged capsules but probably to an enhanced secretion of amorphous viscid material by the organisms. Webster and Burn (1927, a), as regards a mouse *Bacillus enteritidis* strain, found that room temperature (23°) incubation was one factor involved in the transformation to the mucoid type of colony, and that when

TABLE 2

Morphological and biochemical characteristics of E. coli normal S and R dissociates

CULTURES	COLONY MORPHOLOGY, 24 HOURS, 36°C.	ORGANISM MORPHOLOGY			MOTILITY*	PRECIPITATING GROWTH IN PLAIN BROTH, pH 7.2
		Form and size	Capsules	Flagella		
3-S	Domed, smooth, glistening; later mucoid rims; 1 mm. diameter	Single short rods; 2 x 0.7μ	+	+	++	−
3-O_2	Slight convex; rough rim; 0.5 to 2 mm. diameter; occasionally mucoid	Single rods and short chains; 3.0 x 0.7μ	+	+	++	+++
3-R_2	Flat, dry, corrugated surface; thin spreading rim; 6 mm. diameter; never mucoid	Like 3-S; 1.5 x 0.7μ	±	+	±	++
7-S	Like 3-S but non-mucoid; flatter; 1 mm. diameter	Like 3-S	+	+	++	−
7-R_1	Much like 3-R_2; thin, flat, spreading; 3 mm. diameter	Long chains and filaments; 4.4 x 0.6μ	−	+	±	++++
7-R_1 mucoid	Strongly domed, glistening mucoid; 3 mm. diameter; later to 17 mm.	Like 3-S; few filaments	+	+	±	−

* Five-hour plain broth cultures.

Note: All variants hemolyzed rabbit red blood cells alike, also formed acid clot in milk and did not liquefy gelatin.

such mucoid variants were again grown at 37° there was a prompt reversion to a non-mucoid type of colony. Although with this *E. coli*, room temperature incubation following exposure to 36° has favored the development of mucoid rims with 3-S, the mucoid variants of the intermediate types 3-O_2 and 7-R_1 develop this type of colony as readily at 36° as at 22°. The mucoid condition, however, was not entirely stable in any instance.

Differences in bacterial morphology are quite striking (figures 9–15). The normal S and the extreme R stage (3-R_2) are much alike, viz., short, plump, often coccoid single rods with little or no tendency to form chains or filaments in solid or fluid mediums. The mucoid dissociates, from whatever stage they may arise, have also this general morphology. On the other hand, the intermediate phases (O_2 and R_1) tend to grow in chains and in long unsegmented and at times vacuolated filaments. This was particularly true for 7-R_1 (fig. 11). Arkwright (1921) made similar morphological observation as regards the dissociative phases of the typhoid-dysentery group, and Soule (1928) described his R dissociate of *Bacillus subtilis* as producing extremely long filaments. All of these *E. coli* dissociates were flagellated, but only the S and O phases exhibited active motility in 5 hour broth cultures. The flagella (peritrichate) numbered up to 8 for each rod and appeared coarser and longer in the O than the S phases. In the capsulated S and O phases the flagella arise from the outer membrane of the capsule (fig. 13), whereas in the 7-R_1 uncapsulated dissociate, the flagella arise from the cortex of the rod (fig. 14). The capsules stain lightly but distinctly and are particularly easy to demonstrate in the O dissociate phases. The Caesares-Gil stain, of the several tried, gave the most successful preparations. Churchman and Emelianoff (1933) and Maltaner (1934) also have described flagella as arising from the capsule membrane in the typhoid group and thus confirmed the original report of Marrassini (1913). The 7-R_1 dissociate was definitely uncapsulated and the 3-R_2 probably so. O'Neal (1933) described her *E. coli* dissociates as capsulated in the S phase and uncapsulated in the R phase as did also Smith and Bryant (1927) for *E. coli* from calfscours. Our *E. coli* strain also produces, particularly in the O and R phases, an outer zone of amorphous material in appearance suggestive of the "aussenhulle" described by Toennieson (1913) for the Friedlander bacillus. It is apparently a secretory product and seemingly distinct from the capsule as it occurs in cultures of the non-capsulated 7-R_1. As will be shown, the mucoid condition is not necessarily associated with virulence. The mucoid O_2 phase exhibited the full virulence of

the non-mucoid S, but the mucoid R_1 was as devoid of virulence as the non-mucoid. Webster and Burn (1927, b) found the mucoid variant of their standard control S phase of their *B. enteritidis* much the less virulent. Gratia (1921) described a mucoid dissociate of *E. coli* but its relative virulence was not described. This author however, reported less virulence for the S phase than the R, and further his R form was described as actively motile and the S as non-motile. These findings are contrary to those of the writer, and also, as pointed out by Hadley (1927), to the observations of most other investigators of the colon-typhoid group.

As indicated in table 2, the S and early R (O) phases are actively motile. On the other hand, the late R dissociates, although as well equipped with flagella, are almost totally non-motile. Attempts to produce a permanent R dissociate without flagella were unsuccessful, although a temporary loss of flagella in all dissociate phases can be effected by growth on 0.1 per cent phenol agar, as has been the experience of others with the colon-typhoid group.

The normal S phase clouds plain broth without precipitation after 24 hours incubation. With the change to the "rough" phase, even in its early O stages, agglutinating growth with pellicle formation and partial to complete clearing of the broth was noted. This was particularly marked with the 7-R_1 dissociate.

There was no difference in the degree of hemolysis produced between the normal S and its R dissociates. This hemolytic action was well marked on surface streaked rabbit blood plates after five hours incubation at 36°C. Indol production was equal in degree for all the cultures. In milk an acid clot without subsequent peptonization was formed in 24 hours by S and early R's, and in 3 to 5 days by the late R phases. Gelatin was not liquefied. These latter findings are contrary to those reported by O'Neal (1933) for her R_2 and R_3 variants of an encapsulated *E. coli* in that those were reported as producing no change in litmus milk after four days but peptonization after 10 days incubation. Also gelatin was liquefied. Nungester and Anderson (1931) found no distinctive biochemical differences among their series of *E. coli* variants.

The action of this series of *E. coli* dissociates on various fermentable substances are summarized in table 3.

The base medium consisted of 1 per cent Difco peptone, 0.5 per cent NaCl in distilled water (pH 7.2) with brom-cresol-purple indicator added. The test substances were dissolved in 10 per cent amount in distilled water and exposed to 100°C. in the steamer for 10 minutes for

TABLE 3

Fermentation tests

E. COLI DISSOCIATES	GLUCOSE	LEVULOSE	LACTOSE	SUCROSE	MALTOSE	SALICIN	DULCITOL	MANNITOL	GLYCEROL	MANNOSE	XYLOSE	RHAMNOSE	ARABINOSE
3-S	⊕ 1.0	⊕ 1.0	⊕ 2.0	−	⊕ 1.0	⊕ 1.0	⊕ 2.2	⊕ 2.6	⊕ 2.8	⊕ 1.0	⊕ 1.4	⊕ 0.8	⊕ 2.6
3-O_2	⊕ 1.2	⊕ 0.8	⊕ 1.4	−	⊕ 1.0	⊕ 1.4	⊕ 4.0	⊕ 2.4	⊕ 3.8	⊕ 1.3	⊕ 1.9	⊕ 0.8	⊕ 0.8
3-R_2	⊕ 1.6		⊕ 1.5	−	⊕ 1.7	⊕ 1.3	⊕ 3.2	⊕ 2.2	⊕ 4.4	⊕ 1.1	⊕ 2.1	⊕ 0.7	⊕ 1.6
7-S	⊕ 2.0	⊕ 1.3	⊕ 1.0	−	⊕ 3.0	⊕ 1.5	⊕ 2.8	⊕ 1.6	⊕ 2.3	⊕ 1.8	⊕ 2.2	⊕ 1.3	⊕ 1.2
7-R_1	+	+	+	−	+	+	⊕ 1.0	⊕ 2.9	+	+	+	+	+
7-R_1 (mucoid)	+		+	−	+	+	⊕ 2.0	⊕ 1.5	+	+	+	+	+

Five days incubation at 35°C. ⊕ = acidity with gas (lower figures indicate amount of gas in centimeters). + = acidity, no gas. − = no fermentation.

three successive days, and then added in 1 cc. amounts to 9 cc. of the base in test tubes with an inverted 10 mm. diameter inner tube.

As this *E. coli* strain does not split sucrose, but produces acid and gas with lactose and salicin, it conforms to the *Escherichia coli* of the Bergey classification and *Bacillus coli communis* of the Migula nomenclature. The dissociation of strain 3 from the normal S to the extreme R phase was not accompanied by any change, quantitatively or qualitatively, in the action on the 14 test carbohydrates. On the other hand, the 7-R_1 dissociate although form-

ing acid in the same carbohydrates as 7-S, produced gas only with the alcohols, dulcitol and mannitol, and the same was true for its mucoid phase. Penfold (1911) reported that by growing certain *B. coli* strains on a sodium monocholate agar medium he could obtain sub-cultures which acted on his test carbohydrates in a manner similar to the above 7-R_1, and it may be presumed that he was dealing with a similar dissociation phenomenon. Rennebaum (1935) also found that one of his R dissociates from a normal "smooth" *E. coli* failed to produce gas with glucose and was able to show that this failure was due to inability to decompose formates; on the other hand, his most typical R variant split glucose with gas formation but less vigorously than the normal S. Harden and Penfold (1912) had previously found that the fermentative end products of their modified *B. coli* contained less H and CO_2 and more formic acid than that of their normal strains. It would seem then, from the writer's findings and the above literature citations that dissociation from the S to the extreme R phase may or may not involve at certain stages modification or suppression of enzymatic function.

RELATIVE RESISTANCE TO HEAT, BACTERIOPHAGE AND AGING

The only reference to relative degrees of resistance to heat of various dissociates of *E. coli* was found in the study on dissociation of encapsulated bacteria by O'Neal (1933). She reported that the R types exhibited a greater resistance to heat than the normal S. This finding is quite contrary to our experience. There occurred a declining degree of heat resistance with increasing "roughness" with both the mucoid and non-mucoid strains of this *E. coli*.

In these tests, suspensions from 24-hour agar slant growths were made in normal saline to a density of 3 billion organisms. These suspensions were exposed in 11 mm. diameter sealed tubes in a water bath to temperatures ranging from 50° to 60°C. for 15, 30 and 60 minutes. The effect of the exposure in each instance was determined by streaking a 2 mm. loopful of suspension on a purple lactose agar plate. These were examined after 48 hours at 36°C.

There was approximately 4 degrees difference in the killing temperature for the S phase and the respective R dissociate. For example, the highest temperature for 15 minutes survived by 3-S was 56° and for 3-R_2, 52°, and after 1 hour respectively 54° and 50°.

Tests with a strain specific bacteriophage obtained from the intestinal discharge of the patient yielding these *E. coli* strains showed the S phase to be the more sensitive to its lytic action with resistance increasing concomitantly with the development of an increasing degree of "roughness." These findings confirm those of Gratia (1921) and McKinley (1925) for *E. coli*.

Hadley (1927) in his comparative list of characteristics most commonly associated with "normal" and "mutant" types of culture, indicates that the S normal type is generally sensitive to aging and the R mutant type resistant. This was found not to hold for this *E. coli* strain. The advanced R types showed decidedly less resistance to aging, stock cultures of such dissociates dying out at refrigerator temperature in six to ten months, whereas the S would be found alive after a year or longer. The same difference in resistance to aging was noted in broth cultures kept at incubator temperature, S being decidedly the more resistant.

COMPARATIVE RATE OF POPULATION CHANGE IN PLAIN BROTH CULTURES

In table 4, the increase or decrease in number of viable organisms for the S and dissociate phases of this *E. coli* strain while growing in 10 cc. amounts of plain meat infusion, peptone broth, pH 7.2, is expressed in percentage of the bacterial population at various periods in relation to that after one day incubation. Equivalent amounts, suitably diluted, of the broth cultures were surface seeded on lactose purple agar plates and colony counts made. With the normal S the population increased through three days incubation and then gradually decreased until the seventh day of incubation after which there occurred a rapid fall. With 3-O_1 the decrease after one day incubation was gradual and progressive with no reversion toward S, whereas with 3-O_2 a very

abrupt fall occurred in three days with a rise between 5 and 7 days, associated with reversion to S and increase in the viable population nearly to the level of that of one day and then another dissociation with abrupt population decrease. The 7-R_1 culture acted much like 3-O_2 except there occurred no reversion toward S. The 3-R_2 dissociate, as contrasted with 3-S, exhibited a gradual and continued decrease in population beginning within two days. The table also brings out the more prolonged growth energy and viability of the normal S as compared with its dissociates.

TABLE 4

Comparative viability of E. coli dissociates in plain broth. Percentage increase or decrease of viable bacteria from one day growth plating count

(Average of 2 tests)

DISSOCIATES	2 DAYS	3 DAYS	4 DAYS	5 DAYS	7 DAYS	10 DAYS	16 DAYS	21 DAYS	31 DAYS
3-S	+142	+37.1	−7.8	−14.4	−31.2	−81.1	−76.1	−82.4	−85.6
3-O_1	+2.3	−13.2	−30.0	−44.6	−59.7	−78.1	−89.6	−95.2	
3-O_2	+9.4	−62.1	−60.6	−49.1	−14.8	−79.2	−84.8	−90.7	−91.2
7-R_1	+8.9	−61.8	−68.0	−74.8	−79.4	−70.2	−87.0	−69.7	−94.7
3-R_2	−5.2	−28.4	−31.4	−32.5	−48.3	−54.9	−94.8	−91.1	−96.9

E. COLI DISSOCIATION AND VIRULENCE

The studies of Smith and Little (1927), Steinberg and Ecker (1926) and Rennebaum (1935), agree in ascribing the pathogenic effect of *E. coli* culture inoculations into laboratory animals to an exotoxin. It is apparently a capillary poison causing an increased permeability and transudation of vascular fluids particularly into the pulmonary alveoli. On the other hand, Vincent (1930) claimed that both an exotoxin and an endotoxin are concerned and Plantenga (1930) that the toxemia produced in young guinea pigs is dependent on the combined action of an aggressin produced in broth cultures by *E. coli* and the endotoxin of the bacterial bodies. There are very few observations in the literature on the comparative virulence of the normal S phase of *E. coli* and its R dissociates, and apparently none on the several transitional forms in the transformation to the extreme R phase in the colon-typhoid group.

With the first dissociative change from the normal S phase of this *E. coli* strain, there occurred a marked loss of virulence as indicated by intraperitoneal inoculations of white mice, the readily reversible early R stage (3-O_2) exhibiting nearly as complete a loss of virulence as that displayed by the extreme and irreversible R phase (3-R_2).

The minimal lethal dosage determinations were based on the results with white mice of an average 25 grams weight. The intraperitoneal inoculations were generally in 0.5 cc. volume and the results recorded after 2 days. The Berkefeld filtrates of 24-hour plain broth cultures of 3-S were found to have three to four times the toxicity of that of the intermediate (O) phases and the filtrates of the R dissociates, including the mucoid variant of 7-R_1, were practically devoid of toxic effect. A 24-hour broth culture of 3-S, as determined by plating, contains approximately 1.5 billion viable bacilli per cubic centimeter. There were accordingly some 5 million living bacilli in the minimal fatal dosage, 0.0033 cc. As the M.L.D. of a saline suspension from a 24-hour agar slant growth is approximately 50 million viable bacilli, it would appear that the toxin in solution in the broth culture increased the virulence of the organisms about 10 times. On the other hand, in the 0.1 cc. M.L.D. for 3-R_2 broth culture, there were about 150 million viable organisms, or one-fourth the number in the M.L.D. (600 million) of the corresponding saline suspension. Hence it would appear that the growth products of 3-R_2 have some adjuvant effect but not nearly as marked as with the normal S phase. This was also true for the early R variants. It would seem that the presence or absence of a capsule in itself has little or no determinative influence on the virulence of this *E. coli* strain. Thus, 3-O_2, like 3-S, is encapsulated but it exhibited the same lack of virulence as the uncapsulated 7-R_1 under the experimental conditions. The bacterial protoplasm itself also apparently had very little, if any, determinative influence on the lethal effect of either living broth cultures or saline suspensions or on the difference between that of the S and R phases. Saline suspensions, killed by exposure to 60°C. for 1 hour, were lethal on intraperitoneal inoculation only in very large dosage (3 to 4 billion)

for the S phase, or 60 times the dosage of the living suspension, and there was no significant difference between this fatal dosage of the killed S suspension and any of its R dissociates. Differences in the virulence of the S phase and its R variants in this *E. coli* strain seemed thus to be dependent largely on relative capacity for soluble toxin production, and, as will be shown elsewhere, to relative degree of resistance to bacteriolysis within the animal body.

Acquisition of mucoid properties may or may not modify virulence depending upon the phase in which the mucoid condition has developed. Strain 3 in the smooth phase exhibited about the same degree of virulence whether mucoid or non-mucoid. The 7-R_1 mucoid dissociate exhibited little or no greater virulence than the non-mucoid 7-R_1 from which it was derived; on the other hand, the mucoid phase of 3-O_2 exhibited the full virulence of the normal 3-S.

AGGLUTINATION RELATIONSHIPS OF THE S PHASE OF E. COLI AND ITS DISSOCIATES

Although many studies on the antigenic constitution of the S and R phases of *Eberthella typhi*, the *Salmonella* group and *Shigella dysenteriae* are on record, there are very few published observations of this character on *E. coli* and apparently none on the changes in the somatic agglutinogen associated with the transition from the S to the R states.

The experiments reported in tables 5 and 6, permit certain conclusions as regards the antigenic modifications which accompany dissociative changes in this strain of *E. coli*. With this organism, as has been found in general true for the typhoid-paratyphoid-dysentery group by Arkwright (1921), White (1926), Maltaner (1934) and others, dissociation to the R phase involves a change in the body or somatic agglutinogen whereas the flagellar agglutinogen is not modified. However, with this *E. coli* strain, although the intermediate or O stage in the transition to the true R phase exhibits certain of the distinctive cultural and biochemical features of the R phase such as loss of virulence, sedimenting growth in broth cultures, electro-phoretic potential, the somatic

antigenic constitution has been found to be very similar to that of the normal S.

Preparation of immune sera. Rabbits of the same breed and approximate weight were inoculated intravenously with the several dissociated cultures of single cell origin. Immune sera for the whole bacillus and for the deflagellated (soma) organism were prepared. For the former, the antigen consisted of saline suspensions of 24-hour slant cultures heated for 1 hour at 56° to 58°C. and for the latter suspensions which had been heated at 100°C. for one hour and then washed five times with saline by centrifuging. This washing was essential for clear-cut results. The same dosages, intervals between inoculations and number of treatments were employed with each culture, and therefore the findings are directly comparable. The animals were bled seven days after the last of a series of 11 inoculations given over a period of 16 days.

Antigens for agglutination tests. It was necessary to employ a minimum of NaCl in these tests in order to prevent spontaneous clumping of the R suspensions. Accordingly the procedure of Maltaner (1934) was followed. Growths from 24-hour agar slants were suspended in distilled water to a density of 3 billion. As immune serum dilutions were made with an 0.2 per cent saline there was 0.1 per cent in the final mixture. Tests were run with living suspensions, formalinized (0.2 per cent) suspensions and with bacilli heated at 100°C. one hour and washed 5 times with distilled water (deflagellated soma suspension).

Agglutination tests. Equal parts of the bacterial suspension and the serum dilutions (0.5 cc. of each) were mixed and the tubes incubated for 2 hours at 55°C., kept in the refrigerator over night and then read. The first serum dilution showing at least 50 per cent of the bacteria agglutinated was taken as the end point. For type of agglutination the tubes were examined after 30 minutes at 55°C. In the tables the letters F and G indicate respectively floccular and granular agglutination.

Absorptions. The sera were absorbed at a 1:50 dilution, using just enough packed cells in centrifuge tubes to remove the agglutinins from the serum as judged by tests with the homologous strain. The mixtures were incubated at 45°C. for two hours and then centrifuged. The unabsorbed diluted serum was exposed to the same conditions.

In table 5, there are reported agglutination tests with whole bacilli (flagellated) and 100° washed bacilli (deflagellated soma). Deflagellation by exposure to alcohol did not give as satisfactory results as heating.

The results in table 5 show that the flagellar element is antigenically the same in all phases of this *E. coli* strain as has been found to be so generally true for the typhoid and *Salmonella* groups. The whole flagellated bacilli both S, O and R agglu-

TABLE 5

Agglutination tests with E. coli S and dissociates

Serums immune to whole bacilli, heated 56° to 58°C., 1 hour. Test antigens: A = whole living bacilli (flagella and soma). B = bacilli heated 100°C., 30 minutes and washed 5 times (soma).

CULTURES	ANTISERUM TO 3-S		ANTISERUM TO 3-O_2		ANTISERUM TO 3-R_2		ANTISERUM TO 7-R_1		ANTISERUM TO 7-R_1 (MUCOID)	
	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B
3-S	G	G	F	G	F	FG	F		F	FG
	800	1600	3200	1600	1600	1600	800	<100	3200	3200
3-O_2	G	G	F	G	F	FG	F		F	FG
	800	1600	6400	1600	1600	1600	1600	<100	3200	3200
7-S	F	G	F	G	F	FG	F		F	FG
	800	1600	1600	800	1600	1600	800	<100	3200	3200
7-O_2	F	F	F	FG	F	F	F	G	F	F
	6400	12800	6400	3200	25600	6400	1600	100	3200	25600
7-R_1 (mucoid)	<100				<100				<100	
	F	F	F	FG	F	F	F	G	F	F
	3200*	6400	6400	3200	6400*	6400	800	100	3200*	12800
3-R_2	F	G	F	G	F	FG	F	G	F	G
	800	100	3200	1600	1600	3200	3200	1600	1600	100
7-R_1	F	F	F	FG	F	F	F	G	F	F
	1600	400	6400	400	12800	3200	3200	1600	3200	100

F = floccular clumping; G = granular clumping; FG = mixed.

* Formalinized suspension.

tinated to about the same degree in the several sera. The mucoid 7-R_1 generally proved inagglutinable when living suspensions were used but if formalinized the strain agglutinated to the full titer of the serum. The same was true if heated (100°C.) and washed suspensions were used. Either procedure apparently

removed or modified the mucoid element blocking flocculation. This inagglutinability of mucoid *E. coli* variants has been observed by several investigators. Smith and Bryant (1927) reported that a mucoid *E. coli* strain in the S phase would not agglutinate in either S or R serum as did also Nungester and Anderson (1931). Bergstand (1923) also observed that when her D (R) type became mucoid it also became totally inagglutinable. These findings may have been due to the use of living culture suspensions in the agglutination tests.

When the deflagellated (soma) antigens were tested against the whole organism sera, there was little decrease in the titers except that the R cultures agglutinated in low titers with the 3-S and the 7-R_1 (mucoid) sera. In some instances the type of agglutination was floccular, especially with 7-O_2 and 7-R_1 (mucoid), but with the 3-S and 7-S strains it was granular.

There was little difference in the agglutinogenic capacities of the S, O and R variants when the whole organisms were used in immunization. The titers attained seem to be related to the amount of flagellar material and with the O and R variants were particularly high. The antigenic differences between the dissociative phases become clear only when sera immune to the deflagellated (soma) organisms are employed, as illustrated in table 6.

The results in table 6 indicate clearly the antigenic differences of the somas of the S and O groups as contrasted with that of the R. The soma of 3-S and that of 3-O_2 give rise to no agglutinins affecting the R antigens and this is true whether the test is run against the whole unheated bacilli or the boiled and washed organisms (deflagellated). Some degree of somatic difference between 3-S and 3-O_2 is also indicated by the findings in table 6. The serum immune to 3-S soma did not agglutinate the whole bacilli or only in low titer, whereas the boiled and washed (deflagellated) organisms are agglutinated by this serum in very high titer, in fact higher than with the sera immune to the whole organisms. On the other hand, 3-O_2 soma antigen produced a serum agglutinating the whole bacillus antigens to moderate titer as well as the deflagellated antigens in high titer. Accord-

ingly, there would seem to be a soma element present in the intermediate O variant, including 7-R_1 (mucoid), but not in the S which is retained after 100°C. exposure and washing, and which produces agglutinins causing a granular clumping of the whole bacilli S, and a floccular of the whole O variants. The 3-R_2

TABLE 6

Agglutination tests with E. coli S and dissociates

Serums immune to bacilli heated 100°C., 30 minutes and washed 5 times. Test antigens: A = whole living bacilli (flagella and soma). B = bacilli heated 100°C., 30 minutes and washed 5 times (soma).

CULTURES	ANTISERUM TO 3-S		ANTISERUM TO 3-O_2		ANTISERUM TO 3-R_2		ANTISERUM TO 7-R_1		ANTISERUM TO 7-R_1 (MUCOID)	
	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B	Antigen A	Antigen B
3-S	<100	FG 6400	G 200	FG 6400	<100	<100	<100	G 100	G 800	G 1600
3-O_2	G 100	FG 12800	FG 1600	FG 12800	<100	<100	<100	FG 12800	G 3200	FG 1600
7-S	<100	FG 6400	G 400	FG 6400	<100	<100	<100	G 100	G 1600	FG 1600
7-O_2	<100	F 25600	F 1600	F 12800	<100	F 100	F 100	F 200	F 3200	F 12800
7-R_1 (mucoid)	F 400	F 25600	F 1600	F 12800	<100	F 100	FG 100	FG 200	F 6400	F 6400
3-R_2	G 100	<100	G 100	<100	G 800	G 200	F 200	G 3200	F 3200	FG 1600
7-R_1	<100	FG 200	<100	<100	<100	F 200	F 100	G 800	FG 1600	F 1600

F = floccular clumping; G = granular clumping; FG = mixed.

variant, deflagellated by heat, had lost almost entirely its agglutinogenic properties. This soma serum gave a titer of 1:800 for the whole 3-R_2 variant and 1:200 for its soma, whereas the other cultures were practically unaffected. Table 6 shows further that although the soma of 3-R_2 exhibits no relationship to that of the O and S phases, that of 7-R_1 occupies an intermediate position

between the extreme R and the O somas. There is thus presented a gradient series in somatic modification accompaning dissociation. The serum immune to the soma of 7-R_1 (mucoid) is unique in that it agglutinates in fairly high titer the S, O and R cultures, and both the whole bacillus and the soma antigens. It would seem accordingly, that 7-R_1 (mucoid) occupies an intermediate position and combines the antigenic soma elements of the S, O and R phases.

Reciprocal absorption tests resulted in complete absorptions of soma agglutinins from soma sera 3-S, 3-O_2 and 7-R_1 (mucoid) indicating again that the somas of the two latter dissociates have not changed materially from that of the S type. On the other hand, the other R variants absorbed no agglutinins from the S and O soma sera whether whole or deflagellated antigens were used. Further, these tests indicated that although S has a slight R element in its soma protoplasm, R_2 has no S, and the 3-O_2 variant has more of the R element than has 3-S, but on the other hand 7-R_1 (mucoid) has both R and S elements in its soma with the latter strongly predominant and thus indicating a marked degree of reversion toward S.

Maltaner (1934) concluded from a serological investigation of smooth and rough forms of the typhoid bacillus that the smooth somatic antigen responsible for granular agglutination is located in the ectoplasmic structure or capsule. In view of our finding that the S and O phases show capsules and at the same time much heat stable agglutinogen and on the other hand the R phases are non-capsulated, and also nearly devoid of such agglutinogen, it seems evident that Maltaner's conclusion applies to this *E. coli* strain. Further support for this opinion is afforded by the fact that in the reversion of the culture 7-R_1 toward the S phase (7-R_1 mucoid) a capsule is regained and coincidently the serological attributes of the S phase. This heat stable somatic agglutinogen has been related to the specific carbohydrate fractions in the typhoid-paratyphoid group by Furth and Landsteiner (1929), and Dorothea Smith (1927) has reported that in a capsulated *E. coli* strain of bovine origin the capsular substance and the soluble specific substance are apparently identical. Whether

or not in the dissociation of our *E. coli* strain to the R phase, in addition to the loss of this capsular or soluble specific material, the endoplasm undergoes antigenic modification is not clearly indicated by our findings, although probably such is the case. There would seem, however, to be no satisfactory method by which the antigenic properties of the S endoplasm may be compared directly with that of the R due to the interference of the capsular material in the former when tested against R somatic antisera.

SUMMARY

A study of dissociative phenomena exhibited by a capsulated, virulent hemolytic *Escherichia coli* strain of human origin is reported. The experiments were based on cultures derived from a single normal S cell.

An O or intermediate group between S and R phases is described. It is shown that this O group exhibits many of the characteristics of the R phase but reverts readily in fluid medium to S, while the reversion from the R phase is obtained with difficulty, if at all.

Single cells from S and O colonies differ in their dissociative potential, but it was found possible by selective cultural procedures to obtain stabilized pure S organisms.

A mucoid derivative of an R dissociate is described which showed marked reversion toward the S type serologically but not as regards virulence. Mucoid type of growth of S and O cultures was favored by incubation at 22°C., but mucoid derivatives of R developed this type of colony equally well at 22° and 36°C.

As regards fermentative activities, the change from S to the extreme R phase may or may not involve partial suppression of enzymatic function at certain stages, although the power to split any one of the carbohydrates acted on by S was not entirely lost.

The S phase was found more resistant to heat than the R. On the other hand, the R was more resistant to the action of bacteriophage than the S. The S phase exhibited greater growth energy and viability than R.

With the first stage in dissociation toward R, virulence was

greatly decreased, the O stage exhibiting the same low grade of virulence as the extreme R phase. The chief factor in virulence was apparently an exotoxin.

Agglutination and agglutinin absorption tests with this *Escherichia coli* culture indicated:

a. That the flagellar element is antigenically alike in all dissociative phases.

b. That the soma of the intermediate O phase is closely similar antigenically to that of the S.

c. That dissociation to the R phase involves a change in the body or somatic agglutinogen in which the factor of apparent major importance is the loss of a capsule containing the agglutinogen chiefly responsible for the somatic agglutination of the S and O phases. A similar element but with inferior agglutinogenic properties is apparently associated with the endoplasm of the R phase and is specific for that phase.

d. That a mucoid variant arising from an R dissociate acquired a somatic constitution intermediate in type between the S and R phases.

REFERENCES

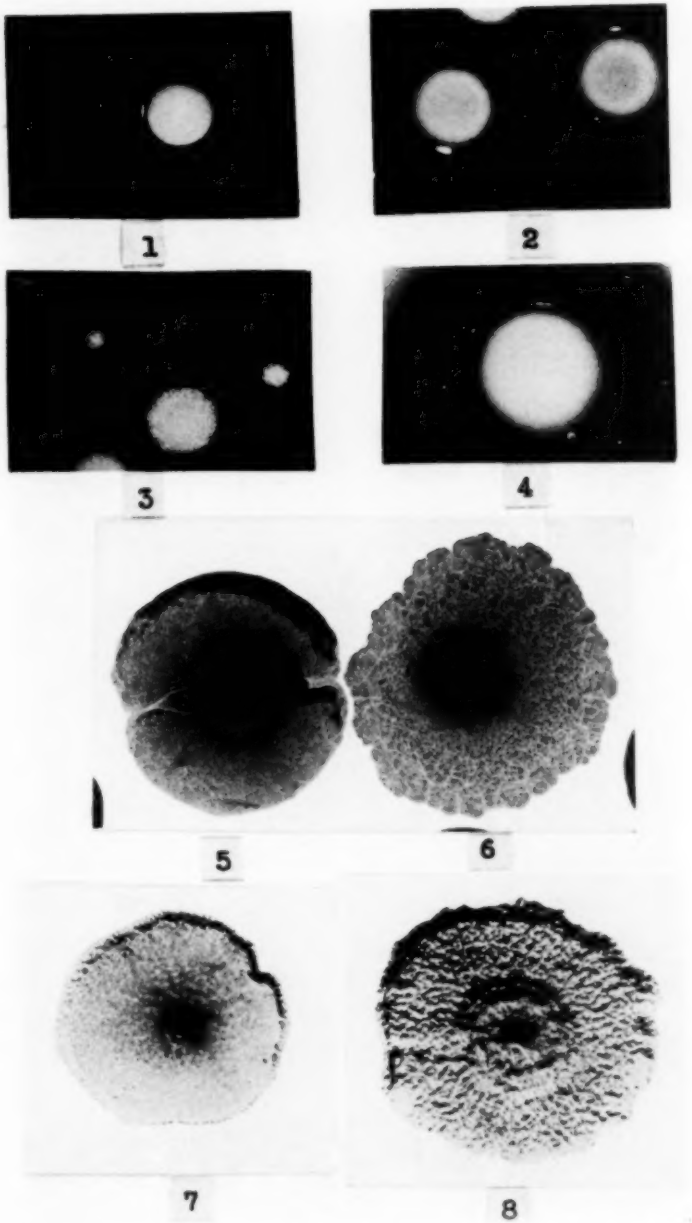
ARKWRIGHT, J. A. 1921 Jour. Path. and Bact., **24**, 36.
BERGSTAND, H. 1923 Jour. Bact., **8**, 173.
CHURCHMAN, J. W., AND EMELIANOFF, V. V. 1933 Jour. Exper. Med., **57**, 485.
DULANEY, ANNA D. 1928 Jour. Infect. Dis., **42**, 574.
FURTH, J., AND LANDSTEINER, K. 1929 Jour. Exper. Med., **49**, 727.
GRATIA, A. 1921 Jour. Exp. Med., **34**, 115.
HADLEY, P. 1927 Jour. Infect. Dis., **40**, 1.
HARDEN, A., AND PENFOLD, W. J. 1912 Proc. Roy. Soc. B., **85**, 415.
KAHN, M. C. 1922 Jour. Infect. Dis., **31**, 344.
MALTANER, F. 1934 Jour. Immunol., **26**, 161.
MARRASSINI, A. 1913 Centralbl. f. Bakt., Orig., **71**, 113.
MCKINLEY, E. B. 1925 Compt. rend. Soc. Biol., **93**, 105.
NUNGESTER, W. J., AND ANDERSON, S. A. 1931 Jour. Infect. Dis. **49**, 455.
O'NEAL, HAZEL E. 1933 Jour. Bact., **26**, 521.
PENFOLD, W. J. 1911 Jour. Hyg., **11**, 487.
PLANTENGA, R. P. B. 1930 Jahrb. f. Kinderheilk., **129**, 253.
RENNEBAUM, E. H. 1935 Jour. Bact., **30**, 625.
SMITH, DOROTHEA 1927 Jour. Exper. Med., **46**, 155.
SMITH, T. AND BRYANT, G. 1927 Jour. Exper. Med., **46**, 133.
SMITH, T., AND LITTLE, R. B. 1927 Jour. Exper. Med., **46**, 123.
SOULE, M. H. 1928 Jour. Infect. Dis., **42**, 93.

STEINBERG, B., AND ECKER, E. E. 1926 Jour. Exper. Med., **43**, 443.
System of Bacteriology, 1924, **4**, 94, Cambridge, England.
TOENNIESON, E. 1913 Centralbl. f. Bakt., Orig. 1, **69**, 391.
VINCENT, H. 1930 Compt. rend. Acad. Biol., **180**, 1624.
WEBSTER, L. T., AND BURN, C. 1927a Jour. Exper. Med., **46**, 855.
WEBSTER, L. T., AND BURN C. 1927b Jour. Exper. Med., **46**, 887.
WHITE, P. B. 1926 Med. Res. Council, Spec. Report Series No. 103.

PLATE 1

COMPARATIVE COLONY MORPHOLOGY OF E. COLI, NORMAL S AND DISSOCIATES

FIG. 1. 3-S, 24 hours, 36°C. ×5.
FIG. 2. 3-S, 24 hours at 36°C. and 24 hours at 23°C. ×5.
FIG. 3. 3-O_2, 24 hours, 36°C. ×5.
FIG. 4. 7-R_1 (mucoid), 48 hours, 36°C. ×5.
FIG. 5. 3-O_1, 24 hours, 36°C. ×20.
FIG. 6. 3-O_2, 24 hours, 36°C. ×20.
FIG. 7. 7-R_1, 48 hours, 36°C. ×5.
FIG. 8. 3-R_2, 48 hours, 36°C. ×5.

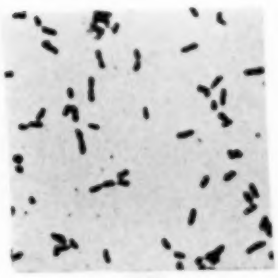


(John C. Torrey and Elizabeth Montu: Variants from single cells of *E. coli*)

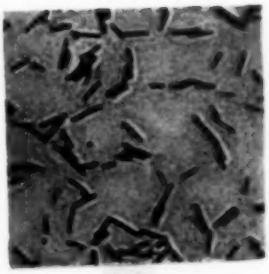
PLATE 2

COMPARATIVE MORPHOLOGY OF E. COLI, NORMAL S AND DISSOCIATES

FIG. 9. 3-S, 24-hour agar slant. ×1200.
FIG. 10. 3-O_2, 24-hour agar slant. ×1200.
FIG. 11. 7-R_1, 24-hour agar slant. ×1200.
FIG. 12. 3-R_2, 24-hour agar slant. ×1200.
FIG. 13. 3-S, 24-hour agar slant. ×3300. Showing capsule with flagella.
FIG. 14. 7-R_1, flagella, but no capsule. ×2300.
FIG. 15. 7-O_2, flagella and capsule. ×2900.



9

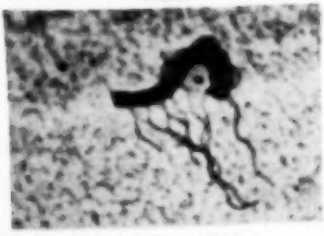


10

11

12

13



14

15

CENTRAL NEW YORK STATE BRANCH

ROCHESTER, NEW YORK, JUNE 17 AND 18, 1936—IN CONJUNCTION WITH THE SUMMER MEETING OF THE AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE

IMMUNO-CHEMISTRY. *Sanford B. Hooker*, Evans Memorial Hospital and Boston University School of Medicine, Boston, Massachusetts.

THE RÔLE OF POLYSACCHARIDES IN IMMUNE REACTIONS. *Forrest E. Kendall*, Presbyterian Hospital, Columbia University, New York, New York.

LIPOIDS OF ANTIPNEUMOCOCCUS SERA. *Kenneth Goodner and Frank L. Horsfall*, Hospital of the Rockefeller Institute, New York, New York.

PROBLEMS IN THE BACTERIOLOGICAL FIXATION OF ATMOSPHERIC NITROGEN AT ORDINARY TEMPERATURES AND PRESSURES. *Caryl P. Haskins*, Research Laboratory, General Electric Company, Schenectady, New York.

A review is presented of investigations of the processes of respiration, oxidation, production of ammonia, and nitrogen fixation in the nitrogen-fixing bacteria, largely those of Burk, Lineweaver, Horner, Milner, Meyerhof, and Deming on *Azotobacter*. The methods of measurement of nitrogen fixed developed by them, and the rôle of calcium and strontium, molybdenum and venadium in nitrogen fixation, as well as the effect upon it of nitrogen and oxygen partial pressures and hydrogen ion concentration are discussed. Consideration is given to the form of the mechanism of nitrogen fixation as derived by Lineweaver, Burk, and Deming. It is suggested that by use of radiational methods and a "sensitive volume" calculation adapted to similar ends by Arnold and Haskins, Haskins, and Haskins and Enzmann, a determination may be made of the effective volume of E, the nitrogen-fixing enzyme "nitrogenase" as defined by Lineweaver, Burk, and Deming. The determination may be made through a study of the variation of $V_{max.}$ as a function of incident x-ray or electronic energy. $V_{max.}$ is defined as a numerical value representing maximum velocity at high nitrogen pressures where the enzyme exists completely in the form N_2E. Work on the problem has been begun at Schenectady.

THEOBALD SMITH; INVESTIGATOR AND MAN. *Simon Gage*, Cornell University, Ithaca, New York.

SOME OF THE PRACTICAL PROBLEMS IN THE SERUM THERAPY OF BACTERIAL INFECTIONS. *Augustus B. Wadsworth*, Division of Laboratories and Research, New York State Department of Health, Albany, New York.

OBSERVATIONS ON PNEUMONIA. *Thomas Ordway*, Albany Medical College, Albany, New York.

CHEMICAL STUDIES ON THE WAX-FRACTIONS OF THE TUBERCLE BACILLUS.

R. J. Anderson, Department of Chemistry, Yale University, New Haven, Connecticut.

The acid-fast bacterial waxes have been found to be esters of higher fatty acids with carbohydrates and certain higher optically active alcohols, hence they differ greatly in chemical composition from waxes of plant or animal origin. Striking differences have been found to exist in the composition of the waxes of the human tubercle bacillus and those of other acid-fast bacteria. The polysaccharides, fatty acids, especially phthioic acid, and a new dihydric alcohol, phthiocerol, $C_{35}H_{72}O_2$, are specific to the human tubercle bacillus wax.

The waxes of the avian tubercle bacillus and the timothy bacillus resemble each other in that they are esters of higher hydroxy acids with trehalose and with two new optically active alcohols, d-eicosanol-2 and d-octodecanol-2. The same alcohols also occur in leprosin, a wax-like substance isolated from the leprosy bacillus.

The principal fatty acid constituents occurring in the bacterial waxes are new and peculiar hydroxy acids of high molecular weight and of unknown chemical constitution. These hydroxy acids are acid-fast.

The bacterial waxes, the hydroxy acids and the optically active alcohols have been found to be remarkable stimulants to cells, especially to fibroblasts and to foreign body giant cells.

FURTHER OBSERVATIONS OF THE TRANSFORMATION OF THE VIRUS OF RABBIT FIBROMA (SHOPE) INTO THAT OF INFECTIOUS MYXOMATOSIS (SANARELLI). *George P. Berry and Helen M. Dedrick*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

In a previous note we have described a method whereby the virus of Rabbit Fibroma (Shope) can be changed into that of Infectious Myxomatosis (Sanarelli). Our original observations have been confirmed and extended. New controls have again attested to the completeness of inactivation of the relatively heat-stable transforming agent derived from myxoma lesions.

Transformation of four strains of fibroma virus has been effected through the use of transforming substances derived from three strains of myxoma, including one carried through cottontail rabbits (*Sylvilagus*) for 34 serial passages. It has been accomplished easily in the domestic rabbit (*Oryctolagus*), with difficulty in the more resistant cottontail, but not at all in immunized (fibroma recovered) rabbits. Transformation has followed the use of heat-inactivated myxoma material only; other viruses, bacteria, heated fibroma suspensions, gastric mucin, and suspensions of mammalian neoplasms, including myxosarcomata, have been ineffective. Rabbits inoculated with fibroma virus and subjected to x-rays and benzol have not developed myxomatosis.

These observations, and those on cross-neutralization tests previously reported, support the view that fibroma virus and myxoma virus are basically related, and that the more virulent myxoma virus is antigenically the more complex.

COEXISTENT INFECTIONS OF INDIVIDUAL CELLS BY MORE THAN ONE FILTERABLE VIRUS. *Jerome T. Sylverton and George P. Berry*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

Rous and Beard have shown that the virus-induced rabbit papilloma of Shope not only possesses the immediate

characters of a tumor, but also gives rise frequently to squamous cell carcinomata. We have confirmed the observations on the papilloma-carcinoma sequence and report that we are in essential agreement with their pathological findings. In certain papillomata, inclusion bodies have been noted by Rous (in press) and by ourselves. Their significance is obscure. The present experiments were undertaken to elucidate their nature after discussions with Dr. Rous and Dr. Rivers. Our object was to study the possibility of superinfection of papilloma cells with extraneous viruses. Seventy-six rabbits, domestic and cottontail, were employed with different combinations of the following viruses; papilloma (Shope), vaccinia, infectious myxomatosis, B virus, Herpes simplex and Virus III.

Our results, based in histopathological findings and on the recovery of all the viruses involved, show that papillomata can readily be superinfected by a variety of viruses and that coexistent infection of individual cells by several viruses can apparently be established. Furthermore, the cells of the squamous cell carcinoma that follows the papilloma can also be parasitized by extraneous viruses. The possibility of encountering non-etiologically significant viruses in virus-induced tumors is emphasized.

GONOCOCCAL INFECTION. 1. DIAGNOSIS BY THE CULTURAL METHOD. 2. FEVER THERAPY. MOTION PICTURES. *Charles M. Carpenter and Stafford L. Warren*, Department of Bacteriology and Department of Medicine (Radiology), School of Medicine and Dentistry, University of Rochester, Rochester, New York.

GENUS STAPHYLOCOCCUS. *David H. Bergey*, Philadelphia, Pennsylvania.

THE SYSTEMATIC RELATIONSHIPS OF THE RED CHROMOGENIC BACTERIA. *Robert S. Breed*, New York State Agricultural Experiment Station, Geneva, New York.

It is proposed that the next edition of the Bergey's Manual limit the species included in the genus *Serratia* Bizio to *Serratia marcescens*, *S. indica* and the two or three additional valid, closely related species now included in Part I of this genus; the remaining species would appear as synonyms.

Species included in Part II are to be removed from the genus. The majority are closely related to or identical with pink to coral red species included by recent authors such as Söhngen, Gray and Thornton and Jensen in *Mycobacterium* or *Proactinomyces*.

While the latter authors have found some peviously described identical or closely related species, there are other identical, or apparently identical, pink to coral red organisms that have been previously described under other names. Many of these may be obtained from culture collections and so can be definitely identified. They are largely non-motile, non-liquefying, Gram-positive to Gram-negative, inert, coccobacteria and are doubtless closely related to certain non-chromogenic soil organisms.

While the relationships of these red chromogens are not yet entirely clear and further study may reveal an earlier generic term, the generic term *Proactinomyces* Jensen (Proc. Linn. Soc. New South Wales, **57**, 1932, 364) appears satisfactory to use for this group until further study clarifies this much tangled situation.

IN WHAT GENUS SHOULD THE SOIL NON-SPORE-FORMING BACTERIA BE PLACED? *H. J. Conn*, New York State Agricultural Experiment Station, Geneva, New York.

Certain very abundant soil bacteria, that are non-spore-forming, normally Gram-negative, and of weak fermentative capacity, are very difficult to place in any system of classification. Three organisms cited here, by way of illustration, are the crown-gall organism, the legume nodule bacteria, and the so-called "radiobacter." Although one is a plant pathogen, one a symbiont with certain plants, and the other a saprophyte, they are almost indistinguishable by cultural tests. The key in Bergey's Manual would suggest placing them in *Achromobacter*, but as the type species of this genus is unrecognizable, it is not possible to be sure that they belong in it. Actually in the 4th edition of this Manual we find: *Phytomonas tumefaciens*, *Rhizobium spp.* (in an entirely different family, the Nitrobacteriaceae), and *Achromobacter radiobacter*.

As similar difficulties are encountered in classifying other soil non-spore-formers, it is recommended that the suggestion of Breed and Conn (J. Bact., 1936, **31**, 517) be followed, of using the temporary genus *Bacterium* for any organisms whose relationships are not yet understood. As an illustration, the combination *Bacterium radiobacter* (Beijerinck and van Delden) Löhnis should be preferred.

THE OXYGEN SUPPLY OF AEROBIC BACTERIA IN LIQUID CULTURES. *Otto Rahn*, Laboratory of Bacteriology, Cornell University, Ithaca, New York.

A WATER SOLUBLE FORM OF ALPHA-NAPHTHOL AND ITS INHIBITIVE ACTION UPON THE INTESTINAL FLORA OF THE HUMAN SUBJECT. *Hubert C. Carel*, Redondo Beach, California.

THE INFLUENCE OF MAGNETISM UPON THE GROWTH OF YEASTS. *Grace Kimball*, Laboratory of Bacteriology, Cornell University, Ithaca, New York.

Experiments have been carried out wherein young yeast cultures were exposed to the magnetic field of 4 Gauss of a permanent horse-shoe or bar magnet for a short period of time. Directly under the magnet there was a noticeable inhibition of budding, usually 20–30 per cent less than the number of buds further away from the magnet. The effect has been observed consistently. When the magnet is shielded by substances which do not affect the magnetic field, such as glass, zinc, or paraffin, the inhibition remains. When the magnetic field is cut off by iron, there is no inhibition.

SOME PROPERTIES OF BACTERIOPHAGE PURIFIED BY ADSORPTION AND ELUTION METHODS. *Leslie A. Sandholzer*, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.

Bacteriophage of high purity has been prepared by a modified Kligler-Olitzki technique in which several concentrations are carried out by the Flosdorf-Mudd method. The resultant dried lytic principle retains its initial activity when kept *in vacuo* for 8 months. The purified dry material has a maximum titer when 0.1 gram is dissolved in 100 cc. of fluid. This titer is often as high as 10^{14}.

No protein is detectable in the purified material by any of the common tests for protein. Micro-Keldjahl determinations yield values of 22 mgm. of nitrogen per gram of dry material or, under optimum conditions of activity, approximately 2.2 mgm. of nitrogen per 100 cc. This value is about one-half that reported by Hosoya, Nagase and Yoshizumi.

When treated with alcohol, acetone,

phenol, lysol or formalin the 0.1 per cent solutions of dry material promptly lose their lytic properties. Lysis occurs only between pH 4.3 and pH 8.8. Exposure for 24 hours to 42°C. inactivates the principle.

Preliminary investigations indicate that the antigenic properties of the purified lytic material are either of a very low order or absent altogether.

STUDIES IN ACID PRODUCTION BY THE ESCHERICHIA-AEROBACTER GROUP OF BACTERIA, AS INDICATED BY DISSOLVED METALLIC IRON. *A. V. Syrocki, James E. Fuller, and R. L. France,* Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

The present paper deals with the application of the dissolved iron test with pure cultures of *Escherichia coli*, *Aerobacter aerogenes*, and a miscellaneous group of Gram-negative lactose fermenting bacteria from water and from salt water fish. Of the strains which were methyl-red positive, 24 or 68 per cent gave positive iron tests. Of the 11 strains which were negative to the iron test, 9 were Aerogenes-like in all tests except the methyl-red. Forty-nine (72 per cent) of the weakly methyl-red positive strains gave negative iron tests. Of the strains which were either negative or questionable to the methyl-red test, 59 (80 per cent) gave negative iron tests. The investigation here reported represents a preliminary study of a method which may be useful as a means of differentiating bacteria of the *Escherichia-Aerobacter* group on the basis of their acid production from carbohydrates.

EXPERIMENTAL PROCEDURE AND DETERMINATION OF PROCESSES FOR CANNED FOODS. *O. B. Williams,* Research Laboratories, National Canners Association, Washington, D. C.

THE INFLUENCE OF CERTAIN FRUITS IN THE DIETS OF RATS ON THE FECAL FLORA AND INTESTINAL REACTION. *W. B. Esselen, Jr.,* Massachusetts State College, Amherst, Massachusetts.

In order to study further the apparent beneficial action of fruit diets for intestinal disorders, work has been carried on to note the effect of cranberry, blueberry and apple diets on the intestinal flora, intestinal putrefaction, and intestinal acidity of the albino rat. It was found that a 20 per cent cranberry diet is effective in reducing the numbers of fecal gas-producing and *B. coli* organisms. The Bergeim ferric oxide reduction test gave evidence that diets of 20 per cent apple, blueberry and cranberry materially decreased intestinal putrefaction. A study of the hydrogen ion concentration of the intestinal contents shows that diets of 10 per cent and 20 per cent raw cranberry and of 100 per cent raw apple significantly increased the acidity of the contents of the cecum and large intestine.

ASCORBIC ACID (VITAMIN C) CONTENT OF LIMA BEANS AS AFFECTED BY SHIPPING, FREEZING AND CANNING. *C. R. Fellers and Walter Stepat,* Massachusetts Agricultural Experiment Station, Amherst, and *G. A. Fitzgerald,* Birdseye Laboratories, Boston, Massachusetts.

The determined protective levels by the Sherman bioassay for vitamin C average considerably higher than those calculated from the 2,6-dichlorophenol-indophenol titration values. The titration method is useful for checking losses in ascorbic acid in lima beans during handling and processing operations.

As judged by the animal bioassay method the protective levels are: market lima beans, 1 to 2 days old, 3 to 3.5

grams; cooked, 3.5 to 4 grams; frozen cooked, 8 grams; reheated canned, 10 grams. Average protective values calculated from the milligrams of ascorbic acid per gram for these products are: 1.7, 4.2, 5.6 and 8.8 grams respectively. Raw, freshly picked lima beans gave a value of 1.04; dried, mature lima beans, trace only.

The vitamin C content of lima beans was reduced as follows: by defrosting frozen beans for 2 to 6 hours, 30 to 40 per cent; by shipment in the pod in iced hampers for 2 days, 30 per cent; by cooking fresh beans, 60 per cent; by cooking frozen beans, 46 per cent.

VITAMIN C IN VEGETABLES. VI. STRING BEANS. *G. L. Mack and D. K. Tressler*, New York State Agricultural Experiment Station, Geneva, New York, and *G. C. King*, University of Pittsburgh, Pittsburgh, Pennsylvania.

STUDIES ON THE PRESERVATION OF GRAPE JUICE. *C. S. Pederson and D. K. Tressler*, New York State Agricultural Experiment Station, Geneva, New York.

PASTEURIZATION OF JUICES OR MUSTS PREPARED FROM SEVERAL VARIETIES OF GRAPES. *C. S. Pederson, E. Arthur Beavens and Harry E. Goresline*, New York State Agricultural Experiment Station, Geneva, New York.

FACTORS THAT INFLUENCE THE SANITARY PRODUCTION OF CRABMEAT. *K. Pierre Dozois*, Department of Bacteriology, University of Maryland School of Medicine, Baltimore, Maryland.

The production of cooked crabmeat, a high perishable and difficult food to produce even under the most watchful supervision, plays an important part in the economic life in the Chesapeake Bay region. Within recent years both Federal and State Health Officials have been greatly concerned with the various problems of the sanitary production of crabmeat. The meat, ready for market, when found to carry fecal *Escherichia coli* is adjudged filthy and a potential danger to the consumer.

Although much has already been accomplished in the way of improving sanitary conditions of the crab plants there still remains the problem of determining more definitely the source of contamination and methods of eliminating it. The lack of sterilization of the crabs during the cooking process may be one important source of contamination. More systematic methods of improving the sanitary working and living conditions of the pickers are of utmost importance. The need of well trained local Sanitary Inspectors and the adoption of enforceable sanitary regulations are greatly needed.

ERRATA

Proceedings of the Maryland Branch, published on page 570 of Volume 31: The name "Earl L. Berkey" should have been "Earl L. Burky".

SONIC ENERGY AS A LETHAL AGENT FOR YEAST AND BACTERIA

T. D. BECKWITH

Department of Bacteriology, University of California, Los Angeles

AND

C. E. WEAVER

Department of Water and Power, Los Angeles

Received for publication July 8, 1936

Crystalline quartz has a certain characteristic which is fundamental to the experiments which we have performed. It is piezo active. Wood and Loomis have described an apparatus which we have utilized with certain modifications and which gives rise to the piezo effect.

With this apparatus we have conducted certain experiments dealing with the killing effect of this source of energy upon bacteria and yeasts. Wood and Loomis (1927) have demonstrated that ultrasonic radiation can disrupt certain higher forms of life. Williams and Gaines (1930) have shown germicidal effect of this wave motion but they used much lower frequency than that which we have applied. Harvey and Loomis (1929) demonstrated the death of luminous bacteria within 1 and 1½ hours when treated by this energy. Chambers and Flosdorf (1936) have utilized a low frequency for extraction of certain fractions of the bacterial body.

The reader is referred to the description of Wood and Loomis for an understanding of the principles involved and of the circuit utilized. We departed from their type in certain respects, as follows. The generator consisted of a circular cast-bronze housing of approximately 20 cm. diameter at the bottom and curved inward to 10 cm. in diameter at the top. One side was grounded. The bottom of this container was of plate glass and upon it was supported a flat-topped mushroom-shaped electrode upon which

rested two quartz crystals, each 3 mm. thick by 5.3 cm. diameter. The dielectric was circulated by means of a pump and was cooled in order to obviate temperature effects upon the microörganisms under test. The power applied to the quartz crystals was high but corona effects were reduced to a minimum by careful selection of a dielectric and by use of the cooling system.

Material to be treated may be placed in a container either entirely of glass, or better still, in a glass tube of 1.25 to 5 cm. in diameter and covered over one end with cellophane. By use of the thin tissue carefully cemented over the glass tube, less loss of energy occurs and the effect of the vibration upon exposed materials is greater in intensity.

EXPERIMENTS WITH YEAST

The yeast which has been used in most instances in this portion of our work has been a strain of *Saccharomyces ellipsoideus*. It has been propagated as needed in grape juice, previously sterilized by the intermittent method. In this medium, luxuriant growth takes place within a few hours and the organism is found to be somewhat larger than the cells constituting the more usual strains utilized by bakers.

Our initial experiments were concerned with the effects of ultrasonic vibration upon cell morphology. It was determined that the cells of rapidly growing yeast were ruptured, following exposure to this form of energy. If a suspension of this yeast in grape juice be treated thus, subsequent microscopic examination shows that most of the field shows merely fragments of cells. Occasionally there remains an intact unit, but if this treated culture now be put back into the incubator, growth is not likely to continue. Evidently, therefore, such cells, although unruptured, must be dead.

Further experiments dealing with this yeast were now carried out with the idea of determining the rate of death when treated with ultrasonic vibration. To make dilution preparations to determine by plating the exact numbers of organisms per unit volume was impracticable; therefore the following technique was adopted. By means of a 6 mm. loop, transfers of somewhat

constant volumes of inoculum may be made repeatedly and a count of resulting colonies following plating upon Sabouraud agar will yield valid indication of a death curve should there be reduction of the numbers of viable organisms. It is realized, however, that this method is not sufficiently refined to yield the most exact data. By use of this technique, however, the following experiment has been performed repeatedly.

The proliferating culture of yeast was placed to a depth of approximately 2.5 cm. in a heavy glass tube with cellophane bottom and this in turn was lowered into the dielectric bath containing the quartz crystal. When in proper position, the cellophane membrane was directly over the disc of the crystal so that the maximum intensity of vibration might be obtained.

TABLE 1

Effectiveness of ultrasonic vibration in killing actively-growing yeast

(Numbers in the volume of a 6 mm. loop)

Before treatment	100,000	28,000	54,000	50,000	36,500
1 minute	17,000	39,000	2,100	1,800	4,200
3 minutes	0	4,300	0	450	8
5 minutes	0	110	0	20	5
10 minutes	0	21	0	8	5
15 minutes	0	0	0	0	5

Treatment of the culture was interrupted at definite time intervals in order to remove a loopful of the yeast suspension for plating, and, following these momentary stops, treatment was resumed until the experiment was terminated.

The results obtained in this portion of the work proved to be variable. Frequently, treatment for a short interval is followed by an increase of the apparent numbers of viable cells and one is led to conclude therefore that the violent agitation first breaks up clumps of cells. In turn, this seeming increase is succeeded by definite lowering of counts as the experiment progresses. In some instances counts dropped precipitously while in other tests the curve of death was more gradual. At random, however, there have been selected five sets of figures which together give a just idea of the effectiveness of this mode of treatment when

applied to yeasts under the conditions outlined. They are presented in table 1.

From these results, it appears that sterilization was complete in 15 minutes in some instances while in others it was partial. All treated materials following the close of the experiments were incubated. In some instances there was no indication that life persisted following irradiation, while in others delayed growth appeared after 4 or 5 days showing that a small number of viable organisms must have persisted, although weakened considerably. In all of these experiments, however, a microscopic examination revealed many disrupted organisms. The conclusion which may be drawn here is that ultrasonic irradiation of this yeast results in death of the cells. The rate of death, however, was variable under the conditions of our work and the causes of such variation were not revealed. It is probable that certain forms may have been sheltered in minute crevices. It is equally possible that spattering on the walls of the culture container deposited some cells temporarily out of the suspending fluid. Either or both probabilities would account for the discrepancies.

Resultant heat as determined by thermometer was of no importance in this outcome since in all instances the final temperature was never higher than 39°C. Wave length may be changed by substituting another crystal of different thickness. Because our power was reduced when thinner crystals were used, it is difficult to evaluate the relationship between lethal effects and wave length. If one assumes a power to be a constant, then this should be inversely proportional to the wave length because of the increased pressure gradient in the standing wave system.

EXPERIMENTS WITH BACTERIA

Certain tests have been made upon bacterial suspensions in tap water to determine whether there is a similar lethal effect when organisms smaller than yeast are exposed to ultrasonic treatment. These have been pursued under three separate headings.

Escherichia coli. For the first group of experiments, a strain of the colon bacillus originally isolated from water was grown for 24 hours in beef-extract broth at 37°. This was then diluted

from 125,000 to 175,000 times with tap water and treated upon five separate occasions and on different days. The treatment period was of five minutes duration with thickness of the quartz disc varying between 1.5 and 4 mm. Counts were made by dilution and plating of each specimen before and after treatment. The results are shown in table 2.

Evidently the vibration rate utilized above is decidedly lethal to the colon bacillus in watery suspension. A further experiment was now conducted to obtain somewhat more exact information regarding the rate of death of these organisms under conditions closely similar. We prepared a suspension of greater concentration of the colon bacillus. The suspension was treated and after 1.5 minutes, the treatment was interrupted for sampling. Treatment was then resumed and continued until an aggregate interval of 5 minutes had elapsed when a final sampling was made. The

TABLE 2

Ultrasonic irradiation of water suspensions of E. coli

Control counts	1,325	3,250	3,700	1,550	1,100
5 minutes treatment	0	0	0	0	0

counts obtained were: Control untreated, 2,000,000 per cubic centimeter; 1.5 minutes, 65,000; 5, minutes, 300. This yields a bactericidal efficiency of slightly less than 100 per cent.

Eberthella typhosa. The second series of experiments dealt with ultrasonic treatment of *E. typhosa* (Hopkins strain), but conditions were not exactly the same as those outlined above. In these experiments, beef-extract broth was placed to a depth of approximately 1.6 cm. in a small flask blown from a Pyrex tube. The diameter was 5 cm. and the bottom was rounded. Thus, a greater portion of the energy was reflected by the contour of the container, and also more was absorbed by the glass than by the cellophane used in the previous experiments. In addition, the suspending medium was now beef-extract broth rather than tap water and as we shall see later, the structure of the suspending medium is of fundamental importance. The preparatory period of incubation at 37° was six hours.

Two experiments were performed under this heading. In each the period of treatment was 10 minutes, and the temperature was 37° at termination of the treatment. In the first, the initial count before irradiation was 20,500,000 per cubic centimeter; and this had diminished to 1,950,000 per cubic centimeter after 10 minutes' exposure. The efficiency therefore was 90.5 per cent. In the second test, the control count was 5,500,000 per cubic centimeter but after 10 minutes' treatment, this had lessened to 12,500 per cubic centimeter—efficiency 99.8 per cent.

From the foregoing, it appears that this type of energy is actively bactericidal both to *E. coli* and to *E. typhosa* in suspensions in water or in beef-extract broth. The particle size which may be disrupted may thus be much smaller than a yeast cell.

Bacillus subtilis. The third division of this portion of the work deals with the effect of ultrasonic radiation upon the spores of *B. subtilis.* The organism originally isolated from soil, was grown upon beef-extract agar for 4 days at 37°. Microscopically, many spores were evident. The culture was washed off with tap water and with constant agitation, the resulting suspension was heated to 80°C. for 5 minutes to kill vegetative forms. With this material, the three following experiments were performed. It should be stated, however, that conditions obtaining throughout this series of experiments were not strictly comparable. Power input was a variable because of differences in crystal thickness. The suspension to be treated was placed in a tube with cellophane diaphragm to a depth of 1.9 cm. in each instance. The period of exposure was 15 minutes. Counts were made by dilution and duplicate plates of the control suspension before treatment and of each of the three aliquot portions following its particular mode of irradiation. These results were obtained:

Experiment 1. The thickness of the crystal was 4 mm. and the final thermometer reading was 15°. Control count, 3,600,000 per cubic centimeter. Following treatment, 1,100,000 per cubic centimeter. Efficiency, 69.4 per cent.

Experiment 2. The thickness of the crystal was 3 mm.; final tempera-

ture 15°. Control count, 3,600,000. Final count following treatment, 1,650,000. Efficiency, 54.2 per cent.

Experiment 3. Same as above except that the thickness of the quartz disc was 1.6 mm. Control count, 3,600,000 and final count 2,050,000. Efficiency 45.8 per cent.

Thermophils. The fourth portion of this work upon bacteria deals with certain experiments in connection with thermophils. It had been demonstrated that vegetative forms and spores within mesophilic range are susceptible to supersonic irradiation. It was now desired to extend these observations to other forms thriving at higher temperature. For this purpose, there was obtained from the laboratories of the California and Hawaiian Sugar Company at Crockett, California, a mixed culture of the organisms causing certain difficulties in refining of cane sugar. The predominant form is a Gram-positive aerobe which grows in chains and which is a luxuriant spore former. Multiplication takes place readily within 48 hours at 50°C. in a solution of brown sugar with concentrations varying between 3 and 20 per cent. These forms, however, are not obligate thermophils inasmuch as multiplication at much slower rate will occur at 37°. With these a variety of tests were made but the following three were most significant. (1) Five minutes' treatment of a suspension in 5 per cent brown sugar produced a reduction from 840 to 15 per cubic centimeter. (2) Upon another occasion, a 5-minute exposure of a culture in 5 per cent brown sugar solution induced a lessening from 16,100 to 2,050 per cubic centimeter. (3) Another culture grown in a 20 per cent brown sugar solution exposed for 5 minutes decreased the count from 3,000,000 to 125,000 per cubic centimeter. All counts were made upon glucose beef-extract agar after 48 hours incubation at 50°.

It appears probable then that ultrasonic radiation is an effective germicide for bacteria when suspended in water, broth, or sucrose solution. Usually Gram-positive organisms are more susceptible to deleterious influences such as germicides than are Gram-negative forms. Each class is killed by this type of radiation. Ultrasonic energy is fatal to bacterial spores likewise.

ANTIBODY AND BACTERIOPHAGE

Ultrasonic energy must kill because of absorption of it by the cell. If the particle be sufficiently small, absorption of energy may not follow. It was desired then to utilize elements of still smaller dimension for exposure. Certain antibodies and also bacteriophage fulfill this requirement. We have found that rabbit antidysentery (Flexner) agglutinating serum following 15 minutes' exposure reveals no difference in titer from the portion which has been untreated. Also, a strain of bacteriophage active against *Salmonella paratyphosum B.* was selected for treatment. This had been "stepped up" after isolation until it had become very active. When this strain of bacteriophage was treated for 10 minutes, we were unable to demonstrate any change in its lytic activity. It seems to be proven, therefore, that these agglutinins and this strain of bacteriophage cannot absorb this type of energy. In spite of this assumption, it should be recognized that certain effects which are chemical in character may be exerted by ultrasonic radiation. The work of Olson and Garden (1932) with bromthymol blue indicates this.

MILK AND ITS BACTERIAL FLORA

The preceding experiments have dealt mainly with particular organisms in a particular environment, and at this point it was determined to widen the field of exploration somewhat so as to include a variety of forms. Cow's milk was selected for this purpose. It contains a flora consisting of a large variety of organisms. Moreover, as a suspending medium it is an emulsion containing lipoid, protein, carbohydrate, together with certain inorganic constituents. The material utilized for this series of over 40 tests was procured from various commercial sources and nearly all of it was certified raw milk. This grade was desired whenever readily available in order to obviate the objectionable feature of the heat treatment of pasteurization, which alters fundamentally the characteristic flora. Following purchase, the milk was allowed to stand at room temperature for lengths of time varying between 2 and 24 hours to permit bacterial multiplication.

Table 3 includes the results of six treatments. These are representative of the large group, but the entire series has not been listed in order to save space. All computations were made from dilutions and poured plates according to the Standard Methods of the American Public Health Association.

It will be noted that table 3 presents results most uneven in character. The point demonstrated, however, is that after exposure of these bacterial forms to such radiation, there is some degree of lessening of bacterial count. Examination of colonies from treated material in comparison with those from control plates did not reveal macroscopical nor microscopical differences. In other words, no selective killing effect toward any particular group was demonstrated.

TABLE 3

Effects of ultrasonic radiation upon bacterial counts in milk

NUMBER	BEFORE TREATMENT	TIME OF TREATMENT	AFTER TREATMENT	EFFICIENCY
		minutes		
1	1,390,000	5	26,000	98.9
2	26,200,000	15	21,000,000	19.8
3	2,200	8.5	50	97.7
4	60,000	10	7,000	88.3
5	3,500,000	10	1,040,000	70.3
6	14,500	6	5,800	60.0

The effectiveness of ultrasonic treatment is shown by these last experiments to be less than when organisms are suspended in water or in physiological saline. Compare tables 2 and 3.

The "dampening" effects of certain components of milk. Bacteria suspended in water are killed by the effects of supersonic energy when this is properly directed and applied. In milk, however, this reduction of numbers of viable bacteria is much less marked. Our next endeavor was to explain this difference. Cow's milk differs from water partly in that it contains lipoid, carbohydrate and protein. We determined to examine the influence of these materials separately.

Attention in this aspect of our experiments was devoted first to possible interference offered by lipoid to the effects of supersonic

vibration. The following were prepared. (*a*) A suspension of olive oil in tap water. This had been emulsified the day previously by ultrasonic treatment and was steamed for 20 minutes. (*b*) An emulsion of butter fat in tap water, steamed for 20 minutes. (*c*) Whole milk steamed for 20 minutes. (*d*) Sterilized tap water. One hundred twenty-five-cubic-centimeter-portions of each were inoculated with 1 cc. of a 1:2000 water dilution of a 24-hour broth culture of the colon bacillus. Enough of each

TABLE 4

Effect of supersonic radiation upon B. coli in lipoid suspensions in water

Water alone and milk served as controls

DESCRIPTION OF SUSPENSION	COUNT PER CUBIC CENTIMETER BEFORE TREATMENT	COUNT PER CUBIC CENTIMETER AFTER TREATMENT	PERCENTAGE EFFICIENCY
Tap water	1,100	0	100
Olive oil in water	940	10	98.9
Butter fat in water	970	0	100
Whole milk	4,000	1,600	60

TABLE 5

Effect of supersonic radiation upon the colon bacillus suspended in 3 per cent solutions of glucose, lactose and sucrose in tap water

MEDIUM FOR SUSPENSION	COUNT PER CUBIC CENTIMETER BEFORE TREATMENT	COUNT PER CUBIC CENTIMETER AFTER TREATMENT
Glucose	825	0
Lactose	850	0
Sucrose	1,350	0
Tap water	1,300	0

preparation to make a layer 2.5 cm. thick was then irradiated for five minutes; after which agar plate counts were made. The results are listed in table 4.

Inspection of table 4 demonstrates that the presence of oil in watery suspension offers no marked degree of resistance to the germicidal effect of supersonic energy.

The possible effect of carbohydrate was next considered. For this purpose there were utilized glucose, lactose and sucrose. Each of these was made up as a 3 per cent solution in tap water

and steamed before use. Tap water alone was included again for control purposes. The method of inoculation and of treatment was precisely the same as that used in the preceding experiment. The results are presented in table 5, from which it is apparent that watery solutions of carbohydrate do not interfere with supersonic germicidal activity.

Attention was next turned to protein as a material which in solution may diminish the germicidal efficiency of supersonic energy. For these experiments, which were two in number, we proceeded in the following manner. There were prepared the following: (*a*) A 0.75 per cent solution of casein in distilled water. The pH of this solution was adjusted to 7.4 and then it was steamed for 20 minutes. (*b*) A 3 per cent solution of Difco

TABLE 6

Effects of supersonic radiation upon the colon bacillus suspended in solutions of casein and of gelatin

Tap water served as a control

SUSPENDING MEDIUM	COUNT PER CUBIC CENTIMETER BEFORE TREATMENT	COUNT PER CUBIC CENTIMETER AFTER TREATMENT	PERCENTAGE OF EFFICIENCY
0.75 per cent casein	2,200	220	90
3 per cent gelatin	2,275	850	62.6
Tap water	1,550	0	100

gelatin in distilled water, steamed for 20 minutes. (*c*) Tap water, steamed for 20 minutes. These were then inoculated with *E. coli* as in the experiments previously described and irradiated. Plating afterwards gave the results which are shown in table 6. Results of one experiment only are indicated, but the second was identical in outcome within the expected limits of experimental error.

Evidently these protein solutions are much more difficult to sterilize by supersonic treatment than are solutions of carbohydrate or suspensions of lipoid. No attempt to date has been made by us to explain the apparent refractoriness of a protein solution to this treatment. Our supposition, however, is that the physical structure of the fluid constitutes the underlying factor.

We have shown here that sonic energy is lethal to yeasts and bacteria under certain conditions. The reasons for this destructive activity are not understood definitely, but we advance the theory that physical factors may underlie this effect. If one assumes a given excitation frequency, f, which is likewise the fundamental frequency of the piezo-crystal; a given velocity of propagation through the material in question, v; then the wave length, λ, is given by the equation:

$$\lambda = \frac{v}{f}$$

With increase in frequency λ becomes smaller, that is, rarefaction and compression approach each other more closely. Therefore the pressure gradient between rarefaction and compression becomes increasingly steeper. We know already that large organisms yield readily to frequencies which are relatively low. To kill bacteria, it has been necessary to increase the frequency for the same power input to the crystal. These facts indicate that compression and its release alternating many times per second produces the killing effect. The problem then becomes one of differential pressure.

We desire to express our thanks to Professor A. R. Olson of the Department of Chemistry at the University of California at Berkeley. It was he who made suggestions which led to these experiments.

SUMMARY

1. A wine yeast, *Saccharomyces ellipsoideus*, suspended in grape juice is usually killed by supersonic treatment.

2. Vegetative organisms of still smaller dimension such as the colon bacillus in tap water, *Eberthella typhosa* in beef broth, or a thermophil in solution of sucrose are killed by this form of energy.

3. Certain particles of still less size such as the agglutinin of *Salmonella paratyphosum B.* or a strain of bacteriophage are not inactivated by supersonic effect.

4. Suspensions of *Bacillus subtilis* spores are diminished greatly in viable numbers by supersonic vibration.

5. The Gram reaction of the organism treated has no influence upon the outcome.

6. The presence of protein in solution interferes with the germicidal activity of supersonic treatment. Lipoid in suspension or carbohydrate in solution does not exert similar depressing effects.

7. Supersonic irradiation of milk is less efficient bactericidally than when applied to fluids containing smaller amounts of protein.

8. It is probable that the pressure gradient constitutes the lethal factor of sonic energy if one assumes viscosity and like factors herein mentioned to be a constant.

REFERENCES

American Public Health Association, Standard Methods of Milk Analysis, 1934.

Chambers, L. A., and Flosdorf, E. W. 1936 Proc. Soc. Exper. Biol. and Med., **34**, 631–636.

Harvey, E. N., and Loomis, A. L. 1929 Jour. Bact., **17**, 373–376.

Olson, A. R., and Garden, N. B. 1932 Jour. Amer. Chem. Soc., **54**, 3617.

Williams, O. B., and Gaines, N. 1930 Jour. Inf. Dis., **47**, 485.

Wood, R. W. 1928 Sci. American, **138**, 201.

Wood, R. W., and Loomis, A. L. 1927 London, Edinburgh and Dublin, Phil. Mag. and Jour. Sci., 7th Ser., **4**, No. 22, 417–436.

A CRITICAL STUDY OF SOME OF THE GROWTH-PROMOTING AND GROWTH-INHIBITING SUBSTANCES PRESENT IN BRILLIANT-GREEN BILE MEDIUM

C. N. STARK AND L. R. CURTIS

Laboratory of Bacteriology, Cornell University, Ithaca, New York

Received for publication March 19, 1936

Ever since the presence of colon organisms has been accepted as an index of contaminated water, bacteriologists have recognized the need for a better medium to detect the presence of these organisms and to inhibit the growth of other gas-producing organisms not known to have any sanitary significance. Jackson (1906) suggested the use of liquid bile for the detection of colon organisms in polluted water. He showed that the bile salts, due to the cholic acid radical, retarded the growth of certain streptococci and destroyed the majority of other bacteria which grow at 37°C. Later Jackson (1907), suggested the addition of 1 per cent lactose to fresh undiluted bile. He also showed that it was possible to desiccate the fresh bile and to use successfully the redissolved material. Hale and Melia (1910), found 10-per-cent bile toxic to colon organisms and recommended the addition of 1-per-cent peptone. Melia (1915), found 5-per-cent bile preferable to 10 per cent. The lower concentration was less toxic to attenuated colon organisms, and induced a more rapid formation of gas.

The next step forward in the development of a suitable culture medium, was the use of dyes. Pfeffer (1886), working with plants, demonstrated the toxic action of aniline dyes. Cornil and Babes (1890), found aniline dyes to be toxic to bacteria. In isolating *Eberthella typhosa* from fecal material, Drigalski and Conradi (1902), used crystal violet to inhibit other bacteria. Churchman (1912), first showed the correlation between the Gram reaction of bacteria and the inhibition of their growth by

gentian violet. He found that the concentration of gentian violet which entirely inhibited the growth of most Gram-positive bacteria, did not inhibit the growth of most Gram-negative bacteria. Hall and Ellefson (1918a), (1918b), first used gentian violet as an inhibitory substance in media for the analysis of water and milk.

Torrey (1913), and Krumwiede and Pratt (1914), used brilliant green in an enrichment medium for the isolation of members of the *Salmonella* and *Eberthella* groups of bacteria. Their work indicated brilliant green to be more toxic to members of the *Escherichia-Aerobacter* group than to species belonging to the *Salmonella* and *Eberthella* genera. Muer and Harris (1920), were first to use brilliant green in a medium for water analysis. They used a 1:10,000 dilution of brilliant green in 5-per-cent dried oxgall. Their results indicated a favorable growth of colon organisms in this medium.

Levine (1922), found concentrations of dried oxgall higher than 2 per cent inhibitory to certain members of the *Escherichia-Aerobacter* group. Winslow and Dolloff (1922), showed that the toxicity of brilliant green to the *Escherichia-Aerobacter* group was materially reduced when brilliant green was added to a medium containing bile. Dunham, McCrady and Jordan (1925) stated that the composition and reaction of the medium exerted a marked influence upon the antiseptic properties of brilliant green. Dunham and Schoenlein (1926) found that a reduced bile content and a higher dilution of brilliant green than was originally suggested by Muer and Harris (1920) gave optimum results for the development of *Escherichia coli*. The composition of their medium was:

	Per cent
Oxgall	2.0
Peptone	1.0
Lactose	1.0
Brilliant green	0.00133

Three different kinds of bacteria which are thought to have no sanitary significance in water are capable of producing gas from lactose. Evidence has been presented by Creel (1914), Winslow (1916), Hall and Ellefson (1918a), and Kahn (1918), to show that sporulating anaerobes have been responsible for positive

presumptive tests which could not be confirmed. False tests caused by aerobic spore-producing bacteria have been reported by Meyer (1918), Ewing (1919), Norton and Weight (1924). Sears and Putnam (1923) and Holman and Meekison (1926) have shown that the combined action of cultures of bacteria, neither culture of which alone is capable of producing gas from lactose, may be responsible for false positive presumptive tests in the analysis of water.

A desirable medium for the qualitative analysis of water would be one which completely inhibited the growth of all organisms responsible for false tests, supported rapid and luxuriant growth of members of the *Escherichia-Aerobacter* groups, and produced large amounts of gas when incubated for only twenty-four hours. The presence of gas in such a medium would indicate sewage contamination and would need no further confirmation.

EXPERIMENTAL

A critical study has been made of the brilliant-green bile medium as recommended by Dunham and Schoenlein (1926) to ascertain how nearly this medium approaches the ideal. The accelerating and inhibiting properties of the component parts of this medium have been tested. The "medium numbers" employed in the tables to follow signify the composition here indicated.

Medium number	*Composition*	*Per cent*
1	Lactose	1
	Peptone	1
2	Lactose	1
	Peptone	1
	Brilliant green	0.00133
3	Lactose	1
	Peptone	1
	Oxgall	2
4	Lactose	1
	Peptone	1
	Oxgall	2
	Brilliant green	0.00133
5	Brilliant green, Difco dehydrated	

All tests were made at least in duplicate and were repeated enough times to convince us of the reliability of the results reported.

The addition of this amount (0.00133 per cent) of brilliant green to lactose-peptone was found to be very toxic to the *Escherichia-Aerobacter* organisms tested. The addition of 2-per-cent oxgall to lactose-peptone increased the rate of growth of the

TABLE 1A

The effect of the constituents of brilliant-green bile medium upon the growth of Escherichia coli, culture 5, isolated from human feces

MEDIA NUMBER	BACTERIA PER 100 CC.	BACTERIA PER CUBIC CENTIMETER			
	0 hours	6 hours	12 hours	24 hours	48 hours
1	4.8	320	22,300,000	360,000,000	355,000,000
2	4.8	*	*	*	*
3	4.8	180	21,150,000	735,000,000	36,500,000
4	4.8	34.5	2,650,000	330,000,000	100,000,000
5	4.8	805	101,250,000	470,000,000	135,000,000

* Less than one per cubic centimeter.

TABLE 1B

The effect of the constituents of brilliant-green bile medium upon the growth of Aerobacter aerogenes, culture 8, isolated from feeds

MEDIA NUMBER	BACTERIA PER 100 CC.	BACTERIA PER CUBIC CENTIMETER			
	0 hours	6 hours	12 hours	24 hours	48 hours
1	11.6	61	12,500,000	265,000,000	25,000,000
2	11.6	*	*	*	*
3	11.6	83	15,500,000	15,000,000	7,500,000
4	11.6	102.5	17,500,000	14,000,000	8,000,000
5	11.6	104.5	26,500,000	30,500,000	19,500,000

* Less than one per cubic centimeter.

cultures of *Escherichia coli* tested, resulting in the presence of many more bacteria per cubic centimeter of medium at the end of twenty-four hours' incubation. After another day's holding the numbers of bacteria had materially decreased (table 1a). Two-per-cent oxgall in lactose-peptone very definitely retarded the growth of the cultures of *Aerobacter aerogenes* tested by us (table

1b). These findings confirm the results of Levine (1922). Two-per-cent oxgall in lactose-peptone containing brilliant green very definitely reduced the toxic action of the brilliant green. These observations confirm the findings of Winslow and Dolloff (1922) and others. Other cultures of *Escherichia-Aerobacter* organisms gave similar results.

TABLE 2A

The effect of the constituents of brilliant-green bile medium upon the production of gas by the lactose-fermenting spore-formers

MEDIA NUMBER	B. AEROSPORUS	B. ARTEROSPORUS	B. TERTIUS	CL. AEROFETIDUM	CL. BUTYRICUM	CL. CHAUVEI	CL. MULTIFERMENTANS	CL. OEDEMATIS-MALIGNI	CL. SPRENGOIDES	CL. WELCHII
1	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−
3	+	−	+	+	−	−	+	+	+	+
4	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−

TABLE 2B

The effect of the constituents of brilliant-green bile medium upon the growth of the synergistic group

MEDIA NUMBER	STREPTOCOCCI FROM FECES AND MILK								STAPH. AUREUS	B. PARATYPHI A	B. PARATYPHI B	B. ENTERITIDIS	PROTEUS VULGARIS	SERRATIA MARCESCENS
	10c1	1d1	8d1	17c1	8b7	8b5	24	21	135					
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	−	−	−	−	−	−	−	−	−	−	+	+	−	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	−	−	+	−	−	+	−	−	+	+	+	+	+
5	+	−	−	+	−	−	+	−	−	+	+	+	+	+

The tests made on the influence of the various constituents of this medium upon the growth of organisms which may be responsible for false tests showed that lactose-peptone as a basic medium did not support the growth of any of the false-test spore-producing organisms studied by us. The addition of oxgall to lactose-peptone produced a medium in which many of the false-

test spore-producing organisms grew. The addition of brilliant green to the lactose-peptone-oxgall medium completely inhibited all of the spore-producing organisms studied (table 2a).

All of the cultures of bacteria used by us which might be responsible for the production of false tests by means of "synergism" grew well in lactose-peptone with or without the addition of oxgall. The addition of brilliant green to lactose-peptone or lactose-peptone-oxgall inhibited certain of these organisms, but failed to inhibit others (table 2b). This fact, it is believed, is a weakness in the brilliant-green medium. Cultures of *Streptococcus* and *Proteus vulgaris* in this medium could produce gas. Bacteria belonging to the *Salmonella* group are able to grow in this brilliant-green bile medium. Cultures of these organisms associated with streptococci might produce a positive presumptive test which could not be confirmed. The presence of such disease-producing organisms, of course, is dangerous. These organisms associated with streptococci, however, could produce a positive presumptive test which could not be confirmed. If the *Salmonella* bacteria were present, members of the *Escherichia-Aerobacter* group would probably also be present.

Since brilliant-green bile medium is being used in the testing of milk for the presence of *Escherichia-Aerobacter* organisms, it was thought to be of value to know the effect upon the growth of false-test organisms of adding to 14 cc. of this medium, 1-cc. amounts of sterile milk. The presence of this additional protein material in the brilliant-green bile medium made possible the growth of certain of the streptococci and some of the spore-producing false-test organisms which were unable to grow in this medium in the absence of this additional protein material (tables 3a and 3b). These findings concerning the reduction of the toxicity of dyes in the presence of additional protein material are in agreement with those reported by Robertson (1908), Kligler (1918), and Stearn and Stearn (1923).

In order to get a clear-cut comparison between the gas-producing properties of members of the *Escherichia-Aerobacter* group when equal numbers of these organisms were added to brilliant-green bile medium from water and milk as sources, further experi-

ments were conducted. The average number of organisms added to each dilution of the triplicate tubes tested, was determined by making colony counts on nutrient agar plates, using 6 plates for each dilution both before and after the tubes were inoculated. About an hour was required to pour the agar plates and inoculate the tubes. Approximately 25 per cent of the plates receiving the smallest number of cells developed no colonies. An approxi-

TABLE 3A

The effect of 1-cc. amounts of milk added to brilliant-green bile medium (Difco) upon the production of gas by the lactose-fermenting spore-formers

MEDIA	B. AEROSPORUS	B. AEROSPOROUS	B. TERTIUS	Cl. AEROFETIDUM	Cl. BUTYRICUM	Cl. CHAUVEI	Cl. MULTIFERMENTANS	Cl. OEDEMATISMALIGNI	Cl. SPHENOIDES	Cl. WELCHII
Brilliant green.....	−	−	−	−	−	−	−	−	−	−
Brilliant green + 1 cc. milk.....	−	−	+	+	−	−	+	−	−	−

TABLE 3B

The effect of 1-cc. amounts of milk added to brilliant-green bile medium (Difco) upon the growth of the synergistic group

MEDIA	STREPTOCOCCI FROM FECES AND MILK								STAPH. AUREUS
	10c1	1d1	8d1	17c1	8b7	8b5	24	21	135
Brilliant green.....	+	−	−	+	−	−	+	−	−
Brilliant green + 1 cc. milk.....	+	+	−	+	+	−	+	−	−

mately similar number of the tubes receiving the highest dilution (smallest number of cells) also showed no growth. No indications of death of cells in the dilution water was observed. These findings indicate a satisfactory agreement between growth in broth and on agar plates by small numbers of cells.

The addition of these amounts of milk to lactose-peptone broth containing brilliant green (medium 2) very definitely reduced the toxic action of the brilliant green. This is shown by the average

amounts of gas in the tubes inoculated from milk, as compared to the complete absence of gas in the tubes inoculated from water with the same numbers of these bacteria. The addition of these amounts of milk to the brilliant-green bile medium as recommended by Dunham and Schoenlein did not affect materially the amounts of gas produced by pure cultures of the *Escherichia-Aerobacter* group (tables 4a and 4b). Similar tests using other *Escherichia-Aerobacter* cultures gave essentially the same results.

TABLE 4A

The effect of the constituents of brilliant-green bile medium upon the production of gas by varying numbers of Escherichia coli, culture 5, isolated from human feces

MEDIA NUMBER	1 CC. DISTILLED WATER CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE						1 CC. SKIMMILK CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE					
	Average number bacteria added											
	106	12	1.2				106	12	1.2			
	Average per cent gas, triplicate tubes											
	24 hours			48 hours			24 hours			48 hours		
1	18	29	27	37	41	41	22	19	17	34	34	36
2	0	0	0	0	0	0	1	2	2	29	25	28
3	43	45	33	52	55	47	39	41	40	51	51	50
4	37	34	29	46	47	46	28	24	21	43	43	39
5	44	32	32	51	48	48	42	36	38	49	45	44

TABLE 4B

The effect of the constituents of brilliant-green bile medium upon the production of gas by varying numbers of Aerobacter aerogenes, culture 8, isolated from feeds

MEDIA NUMBER	1 CC. DISTILLED WATER CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE						1 CC. SKIMMILK CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE					
	Average number bacteria added											
	70	7	1.2				70	7	1.2			
	Average per cent gas, triplicate tubes											
	24 hours			48 hours			24 hours			48 hours		
1	23	17	0	47	52	0	22	14	5	50	50	40
2	0	0	0	0	0	0	10	3	2	40	37	37
3	21	11	3	60	58	80	18	12	1	43	47	40
4	26	17	0	53	46	0	12	3	3	37	37	17
5	27	23	15	55	57	47	33	12	10	52	55	50

SUMMARY

The use of 0.00133 per cent of brilliant green in 1-per-cent peptone and 1-per-cent lactose completely inhibited the growth of all members of the *Escherichia-Aerobacter* group, tested by us.

The addition of 2-per-cent dried oxgall to 1-per-cent peptone and 1-per-cent lactose very definitely accelerated the growth of the members of the species of *Escherichia* studied. This amount of dried oxgall retarded slightly the growth of the cultures of *Aerobacter aerogenes* used in these experiments. The toxicity of 0.00133 per cent of brilliant green is very materially reduced by the addition of 2 per cent dried oxgall to 1-per-cent peptone and 1-per-cent lactose.

Two-per-cent dried oxgall added to 1-per-cent peptone and 1-per-cent lactose supported the growth of certain sporulating aerobic and anaerobic bacteria which may be responsible for false tests. The addition of 0.00133 per cent brilliant green to 1-per-cent peptone, 1-per-cent lactose, and 2-per-cent dried oxgall, completely inhibited the growth of all the false-test sporulating bacteria tested by us.

The addition of 0.00133 per cent of brilliant green to 1-per-cent peptone and 1-per-cent lactose or 1-per-cent peptone, 1-per-cent lactose and 2-per-cent dried oxgall failed to inhibit the growth of certain cultures of bacteria which could be responsible for false tests by means of "synergism."

That additional protein material definitely reduces the toxic action of brilliant green, in the brilliant-green bile medium, was demonstrated by the addition of 1-cc. amounts of sterile milk to 14 cc. of the medium. This additional amount of protein material resulted in the growth of some of the sporulating, false-test organisms, and in the growth of an even larger number of the cultures which may be responsible for false tests by means of "synergism." These findings have special significance in the use of brilliant-green bile medium to test milk for the presence of members of the *Escherichia-Aerobacter* group.

These studies, it is believed, further emphasize the dangers involved in the use in culture media of any inhibitory substance, the toxicity of which is influenced by the amount of protein and the reaction of the medium.

REFERENCES

CORNIL AND BABES 1890 Cited by STILLING, J. Anilin Farbstoffe als Antiseptica und ihre Anwendung in der Praxis. I. Mittheilung. Strassburg.

CHURCHMAN, JOHN W. 1912 Jour. Exper. Med., **16**, 221.

CREEL 1914 U. S. Hygiene Lab. Bull. 100.

DRIGALSKI, V., AND CONRADI, H. 1902 Zeitschr. f. Hyg., **39**, 283.

DUNHAM, H. G., MCCRADY, M. H., AND JORDAN, H. E. 1925 Jour. Amer. Water Works Assoc., **14**, 535.

DUNHAM, H. G., AND SCHOENLEIN, H. W. 1926 Stain Tech., **1**, 129.

EWING, C. LEROY 1919 Amer. Jour. Pub. Health, **9**, 275.

HALE, FRANK E., AND MELIA, THOMAS W. 1910 Jour. Inf. Dis., **7**, 587.

HALL, IVAN C., AND ELLEFSON, L. J. 1918a Jour. Bact., **3**, 329.

HALL, IVAN C., AND ELLEFSON, L. J. 1918b Jour. Bact., **3**, 353.

HOLMAN, W. L., AND MEEKISON, D. M. 1926 Jour. Inf. Dis., **39**, 145.

JACKSON, DANIEL D. 1906 Biological Studies by the Pupils of W. T. Sedgwick.

JACKSON, DANIEL D. 1907 Jour. Inf. Dis., Supplement No. 3, 30.

KRUMWIEDE, CHARLES, JR., AND PRATT, JOSEPHINE S. 1914 Jour. Exper. Med., **19**, 501.

KAHN, R. L. 1918 Jour. Bact., **3**, 561.

KLIGLER, I. J. 1918 Jour. Exper. Med., **27**, 463.

LEVINE, MAX 1922 Jour. Amer. Water Works Assoc., **9**, 612.

LISK, H. 1923 Jour. Amer. Water Works Assoc., **10**, 139.

MELIA, THOMAS W. 1915 Amer. Jour. Pub. Health, **5**, 1168.

MUER, T. C., AND HARRIS, R. L. 1920 Amer. Jour. Pub. Health, **10**, 874.

MEYER, C. M. 1918 Jour. Bact., **3**, 9.

NORTON, J. F., AND WEIGHT, J. J. 1924 Amer. Jour. Pub. Health, **9**, 273.

PFEFFER 1886 Quoted by SIMON AND WOOD. Amer. Jour. Med. Sc., 1914, **147**, 247.

ROBERTSON, T. B. 1908 Jour. Biol. Chem., **4**, 32.

STILLING, J. 1890 Anilin Farbstoffe als Antiseptica und ihre Anwendung in der Praxis. I. Mittheilung. Strassburg.

SEARS, H. J., AND PUTNAM, JOHN J. 1923 Jour. Inf. Dis., **32**, 270.

STEARN, ESTHER W., AND STEARN, ALLEN E. 1923 Jour. Bact., **8**, 567.

TORREY, J. C. 1913 Jour. Inf. Dis., **13**, 263.

WINSLOW, C.-E. A., AND DOLLOFF, A. F. 1922 Jour. Inf. Dis., **31**, 302.

WINSLOW, C.-E. A. 1916 Jour. Amer. Water Works Assoc., **3**, 927.

INCREASED GROWTH AND GAS PRODUCTION BY ESCHERICHIA-AEROBACTER ORGANISMS IN BRILLIANT GREEN BILE MEDIUM CONTAINING SODIUM FORMATE

C. N. STARK AND L. R. CURTIS

Laboratory of Bacteriology, Cornell University, Ithaca, New York

Received for publication March 17, 1936

Culture media for the detection of *Escherichia-Aerobacter* organisms in water and milk have undergone many changes in an attempt to find a satisfactory medium. Bile, buffers, and various dyes have been incorporated to improve the media. Brilliant-green lactose peptone bile (Dunham and Schoenlein, 1926) is an improved medium which has been tested (Stark and Curtis, 1936) to determine its toxicity to small numbers of *Escherichia-Aerobacter* organisms and its ability to inhibit the growth of the various organisms responsible for false tests. Stark and England (1935) used 0.5-per-cent sodium formate to accelerate growth, to stimulate production of gas, and to increase the amount of gas produced. Since it seemed desirable to know whether 0.5-per-cent sodium formate added to the brilliant-green bile medium would improve it, this study was made.

EXPERIMENTAL

Five-tenths per cent sodium formate has been added to brilliant-green bile medium and its various constituents to determine its influence on growth and gas production by members of the *Escherichia-Aerobacter* groups and to learn the capacity of these media to inhibit the growth of the various organisms responsible for false tests. The "medium numbers" employed in the tables to follow signify the composition here indicated.

Medium number	Composition	Per cent
1	Lactose	1
	Peptone	1
2	Lactose	1
	Peptone	1
	Na formate	0.5
3	Lactose	1
	Peptone	1
	Oxgall	2
4	Lactose	1
	Peptone	1
	Oxgall	2
	Na formate	0.5
5	Brilliant green, Difco dehydrated	
6	Brilliant green, Difco dehydrated	
	Na formate	0.5

All tests were made at least in duplicate and were repeated enough times to convince us of the reliability of the results reported.

It was found that the addition of 0.5-per-cent sodium formate to the complete brilliant-green bile medium or to any of its constituents tended to increase the rate of growth of the *Escherichia-Aerobacter* organisms and to result in the presence of a larger number of organisms (tables 1a and 1b). Other cultures of *Escherichia-Aerobacter* organisms gave similar results.

The addition of 0.5-per-cent sodium formate to the complete brilliant-green bile medium or to any of its constituents did not materially influence the growth of organisms which may be responsible for false tests (tables 2a and 2b).

The influence of 1-cc. amounts of sterile milk in the complete brilliant-green bile medium and its various constituents (Stark and Curtis, 1936) showed that amounts of protein, which would of necessity be added when milk is tested, are sufficient to decrease definitely the inhibitory action of the brilliant green upon certain organisms responsible for false tests. The addition of 0.5 per cent sodium formate to complete brilliant-green bile medium or to

any of its constituents, in the presence of 1-cc. amounts of sterile milk added to 14 cc. of these media, was found to have little influence upon the growth of false test organisms. There was a slight tendency, however, for the medium containing sterile milk and formate to be more toxic (tables 3a and 3b).

TABLE 1A

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the growth of Escherichia coli, stock culture 71

MEDIA NUMBER	BACTERIA PER 100 CC.	BACTERIA PER CUBIC CENTIMETER			
	0 hours	6 hours	12 hours	24 hours	48 hours
1	18.4	42.5	850,000	160,000,000	410,000,000
2	18.4	65.5	1,865,000	1,145,000,000	1,845,000,000
3	18.4	124.5	3,480,000	395,000,000	88,500,000
4	18.4	58.0	2,400,000	665,000,000	1,245,000,000
5	18.4	57.0	1,420,000	475,000,000	110,000,000
6	18.4	112.5	1,370,000	535,000,000	1,020,000,000

TABLE 1B

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the growth of Aerobacter aerogenes, no. 8, isolated from feeds

MEDIA NUMBER	BACTERIA PER 100 CC.	BACTERIA PER CUBIC CENTIMETER			
	0 hours	6 hours	12 hours	24 hours	48 hours
1	11.6	61	12,500,000	265,000,000	25,000,000
2	11.6	82.5	16,500,000	610,000,000	121,500,000
3	11.6	83	15,500,000	15,000,000	7,500,000
4	11.6	169	13,000,000	270,000,000	244,000,000
5	11.6	102.5	17,500,000	14,000,000	8,000,000
6	11.6	16	11,000,000	175,000,000	101,500,000

To obtain comparisons of gas production by equal numbers of members of the *Escherichia-Aerobacter* group inoculated from water and from milk into the constituents of brilliant-green bile medium with and without sodium formate, further experiments were conducted. The average number of organisms added to each dilution of the triplicate tubes tested was determined by making colony counts on nutrient agar plates, using 6 plates for

each dilution both before and after the tubes were inoculated. More than an hour was required to pour the agar plates and inoculate the tubes. Similar numbers of broth tubes and agar plates receiving the highest dilution (smallest number of cells)

TABLE 2A

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the production of gas by the lactose-fermenting spore-formers

MEDIA NUMBER	B. AEROSPORUS	B. ASTEROSPORUS	B. TERTIUS	CL. AEROFETIDUM	CL. BUTYRICUM	CL. CHAUVEI	CL. MULTIFERMENTANS	CL. OEDEMATIS-MALIGNI	CL. SPHENOIDES	CL. WELCHII
1	−	−	−	−	−	−	−	−	−	−
2	+	−	+	−	−	−	+	−	−	−
3	+	−	+	+	−	−	+	+	+	+
4	−	−	+	+	−	−	+	+	+	+
5	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−

TABLE 2B

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the growth of the synergistic group

MEDIA NUMBER	STREPTOCOCCI FROM FECES AND MILK								STAPH. AUREUS	S. PARATYPHI A	S. PARATYPHI B	S. ENTERITIDIS	PROTEUS VULGARIS	BERRATIA MARCESCENS
	10c1	1d1	8d1	17c1	8b7	8b5	24	21	135					
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	−	−	+	−	−	+	−	−	+	+	+	+	+
6	+	−	−	+	−	−	+	−	−	+	+	+	+	+

showed no growth. A satisfactory agreement between growth in broth and on agar plates was obtained.

It has been previously observed (1936) that the addition of 1-cc. amounts of sterile milk to lactose peptone broth containing brilliant green, decreased the toxic action of the brilliant green;

and that the addition of this amount of milk to the complete brilliant-green medium, did not affect materially the amounts of gas produced by pure cultures of the *Escherichia-Aerobacter* group. In all tests here reported, the addition of 0.5 per cent sodium formate to the complete brilliant-green bile medium or any of its

TABLE 3A

The effect of 1-cc. amounts of milk added to brilliant-green-bile-sodium formate medium upon the production of gas by the lactose-fermenting spore-formers

MEDIA	B. AEROSPORUS	B. AEROSPORUS	B. TERTIUS	CL. AEROFETIDUM	CL. BUTYRICUM	CL. CHAUVEI	CL. MULTIFERMENTANS	CL. OEDEMATIS-MALIGNI	CL. SPHENOIDES	CL. WELCHII
Brilliant green, Difco dehydrated	−	−	−	−	−	−	−	−	−	−
Brilliant green, Difco dehydrated + 1 cc. milk	−	−	+	+	−	−	+	−	−	−
Brilliant green, Difco dehydrated + Na formate 0.5 per cent + 1 cc. milk	−	−	+	+	−	−	+	−	−	−

TABLE 3B

The effect of 1-cc. amounts of milk added to brilliant-green-bile-sodium formate medium upon the growth of the synergistic group

MEDIA	STREPTOCOCCI FROM FECES AND MILK								STAPH. AUREUS
	10c1	1d1	8d1	17c1	8b7	8b5	24	21	135
Brilliant green, Difco dehydrated	+	−	−	+	−	−	+	−	−
Brilliant green, Difco dehydrated + 1 cc. milk	+	+	−	+	+	−	+	−	−
Brilliant green, Difco dehydrated + Na formate 0.5 per cent + 1 cc. milk	+	−	−	−	−	−	+	−	−

constituents resulted in an earlier production of gas and in the formation of two to three times as much gas as was produced in similar media which did not contain sodium formate (tables 4a and 4b). Similar tests using other *Escherichia-Aerobacter* cultures gave essentially the same results.

TABLE 4A

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the production of gas by varying numbers of Escherichia coli, no. 5, isolated from human feces

MEDIA NUMBER	1 CC. DISTILLED WATER CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE						1 CC. SKIMMILK CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE					
	Average number bacteria added						Average number bacteria added					
	106	12	1.2				106	12	1.2			
	Average per cent gas, triplicate tubes						Average per cent gas, triplicate tubes					
	24 hours			48 hours			24 hours			48 hours		
1	18	29	27	37	41	41	22	19	17	34	34	36
2	57	58	33	100	100	100	70	79	74	100	100	100
3	43	45	33	52	55	47	39	41	40	51	51	50
4	75	71	66	100	100	100	78	62	50	100	100	92
5	44	32	32	51	48	48	42	36	38	49	45	44
6	90	80	75	100	100	100	89	70	41	100	100	100

TABLE 4B

The effect of the constituents of the brilliant-green-bile-sodium formate medium upon the production of gas by varying numbers of Aerobacter aerogenes, no. 8, isolated from feeds

MEDIA NUMBER	1 CC. DISTILLED WATER CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE						1 CC. SKIMMILK CONTAINING DIFFERENT NUMBERS OF BACTERIA WAS ADDED TO EACH TUBE					
	Average number bacteria added						Average number bacteria added					
	70	7	1.2				70	7	1.2			
	Average per cent gas, triplicate tubes						Average per cent gas, triplicate tubes					
	24 hours			48 hours			24 hours			48 hours		
1	23	17	0	47	52	0	22	14	5	50	50	40
2	59	59	32	100	100	100	67	43	34	100	100	93
3	21	11	3	60	58	80	18	12	1	43	47	40
4	58	41	18	100	100	65	57	23	13	100	100	93
5	27	23	15	55	57	47	33	12	10	52	55	50
6	85	49	44	100	100	100	68	54	14	100	100	100

SUMMARY

It has been shown that brilliant-green bile medium as now used, does not inhibit the growth of all bacteria which may be responsible for false tests in the analysis of water and milk.

The addition of 0.5 per cent sodium formate to brilliant-green bile tended to increase the rate of growth of *Escherichia-Aerobacter* organisms and resulted in the presence of a larger number of organisms.

Five-tenths per cent sodium formate added to brilliant-green bile caused an earlier production of gas and the formation of two to three times as much gas.

The addition of 0.5 per cent sodium formate did not materially influence the growth of organisms which may be responsible for false tests.

REFERENCES

DUNHAM, H. G., AND SCHOENLEIN, H. W. 1926 Stain Tech., **1**, 129.
STARK, C. N., AND ENGLAND, C. W. 1935 Jour. Bact., **29**, 26.
STARK, C. N., AND CURTIS, L. R. 1936 Jour. Bact., **32**, 375.

SUBSTITUTES FOR POTASSIUM IN METABOLISM OF THE LOWEST FUNGI

OTTO RAHN

Bacteriological Laboratory, New York State College of Agriculture, Cornell University, Ithaca, New York

Received for publication April 6, 1936

Though textbooks on plant physiology, animal physiology and biochemistry state that potassium is necessary for all organisms, the rôle of this element in the living cell is still unknown. So far as the hyphomycetes and bacteria are concerned, it is known that rubidium and possibly cesium, may take the place of potassium. There is also considerable evidence that most of the Gram-negative bacteria thrive well without potassium (table 1).

The author repeated these tests with 3 Gram-positive and 3 Gram-negative organisms. The medium consisted of 1.88 gram NH_4Cl, 1.0 gram $Na_2HPO_4 \cdot 12H_2O$, 0.2 gram $CaCl_2$ and 50 grams glucose per liter. To this medium, increasing amounts of KCl were added, from 0.005 to 0.100 per cent. There was good growth and equal turbidity without and with potassium in the cultures of *Escherichia coli*, *Serratia marcescens* and *Pseudomonas fluorescens*. With *Bacillus subtilis*, however, there was no growth without potassium, and good growth even with the smallest amount of this ion. *Azotobacter chroococcum* and *Micrococcus aureus* did not grow in this medium. In order to be quite certain of not transferring any potassium with bacteria, new cultures with and without K were inoculated from the cultures without K. Most of these cultures were cloudy in twenty-four hours, and plate counts were made after forty-eight hours. Table 2 shows that the Gram-negative bacteria were not greatly favored by the presence of potassium. The numbers might well be considered final crops. Since these organisms grew to such numbers after 3 transfers in K-free medium, there can be no doubt that their

K-requirement is extremely low if they need this ion at all. Contrasted with this is the Gram-positive *Bacillus subtilis* which

TABLE 1

Literature references on potassium requirements

	K NECESSARY	K CAN BE SUBSTITUTED BY					
		Na	Li	Rb	Cs	U	Ra
Aspergillus niger							
1870 Raulin	+						
1882 Naegeli	+	0		+	+		
1895 Benecke	+	0		+*	+*		
1912 Sauton	+	0	0	+†	0		
Mycobacterium tuberculosis							
1912 Sauton	+	0	0	+†	0		
1913 Löwenstein	0						
1913 Besancon, Philbert and Boudin	+	0					
1919 Lockemann	+	0					
1926 Frouin and Guillaumie	+	0		0	0		
1928 Boissevain	+	0	0	+	0	0	0
Bacillus subtilis							
1918 Koch	+	0					
1928 Boissevain	+	0	0	+	0	0	0
Azotobacter							
1901 Gerlach and Vogel	0						
1932 Waksman	+	0					
Rhizobium							
1932 Fred	+	0					
Colon-typhoid group							
1905 Cache	0						
1916 Zunz and Györgi	0						
1928 Boissevain	0						
Pseudomonas pyocyanea							
1916 Zunz and Györgi	0						
Vibrio cholerae							
1916 Zunz and Györgi	0						
Staphylococcus aureus							
1916 Zunz and Györgi	+(?)						

* Formation of spores poor or absent.

† Crop is decreased, but cells are normal.

did not grow at all in the K-free medium. This was tested again in 6 transfers at different times, always with the same result.

It must be kept in mind that 1 mgm. of bacterial solids of

Bacterium coli amounts to 4.5×10^9 cells. This is almost exactly the amount in 100 cc. of the above cultures. Probably, less than 10 per cent of this is ash, and the K content in the bacteria from 100 cc. of culture would be less than 0.05 mgm. Such small amounts might perhaps come from the glass, or from impurities of the chemicals. We can state only that by using analyzed C.P. chemicals in glass vessels, most Gram-negative bacteria can grow without addition of K to the medium while Gram-positive aerobic sporeformers like *B. subtilis* require the addition of K for growth.

The interest in the rôle of K was revived when Zwaardemaker (1921) showed that isolated frog hearts transfused with Ringer solution stopped beating if the potassium was omitted, but started again when irradiated by radium, or when the K of the

TABLE 2

Cells per cubic centimeter after two days' incubation at 30° in potassium-free medium

	WITHOUT ADDITION	WITH 0.005 PER CENT KCl	WITH 0.100 PER CENT KCl
Escherichia coli	38,000,000	51,000,000	18,000,000
Serratia marcescens	65,000,000	60,000,000	92,000,000
Pseudomonas fluorescens	40,000,000	63,000,000	62,000,000
Bacillus subtilis	100	2,100,000	1,200,000

Ringer solution was replaced by radioactive equivalents of Rb, Th, or U. Boissevain (1928) showed quantitatively, by crop measurements, that in the tubercle bacterium and other mycobacteria, K could be replaced by Rb with only a slight decrease in crop, but that Cs, Li, U, or Ra were entirely insufficient.

Regarding the potassium requirement of yeast, there are many citations stating that this element is necessary, but no experiments to prove this assertion could be found. If the rôle of potassium in yeast were merely the provision of radiation, as in the case of the beating frog heart, the radiation could be provided by surrounding a potassium-free yeast culture with a large amount of potassium salts. This possibility was tested with a top yeast isolated from bakers' yeast, which grew well in the above-mentioned medium with potassium. The potassium-free culture

received beta-radiation through a cellophane window[1] in a pasteboard box containing about 30 grams of KCl. The entire box, with the exception of the cellophane window, had been paraffined to prevent any traces of KCl-dust from getting into the medium. All cultures were grown in Petri dishes in order to have the source of radiation within 1 cm. of the surface of the culture. The cellophane window was large enough to permit irradiation of the entire surface. Of the two controls without potassium, one had a cellophane cover without KCl, the other a glass cover. Two other cultures received small amounts of KCl.

Table 3 shows considerable growth even in the controls, the potassium being provided partly from the glass and partly from the inoculum. Potassium, however, was at the minimum be-

TABLE 3

Yeast growth in media without and with potassium, and without and with potassium irradiation (cells of yeast per cubic centimeter)

	GLASS CONTROL, NO KCl	CELLOPHANE CONTROL, NO KCl	IRRADIATED, NO KCl	0.4 MGM. KCl IN 100 CC.	1.0 MGM. KCl IN 100 CC.
At start	25,700	25,700	25,700	92,000	15,800
After 1 day	374,000	480,000	515,000		3,800,000
After 2 days	1,900,000	685,000	790,000	7,900,000	16,500,000
After 3 days	1,850,000	1,520,000	1,250,000	8,000,000	19,500,000

cause even as small a quantity as 0.4 mgm. KCl per 100 cc. made the crop 5 times as large. The irradiation with beta-rays had not the least stimulating effect upon the yeast. We must conclude, therefore, that potassium is necessary for yeast growth, and that its rôle is not exclusively limited to its radio-activity.

The same result was obtained when potassium was replaced by other radio-active elements, using radio-active equivalents instead of molecular equivalents as suggested by Zwaardemaker. Medium and test organism were the same as above, except that potassium was more successfully eliminated. The radio-active

[1] The cellophane used in this experiment transmitted about 90 per cent of the beta-radiation of potassium, according to measurements by Dr. D. F. Weekes, of the Physics Department of Cornell University.

substances were added before sterilization. Table 4 shows the concentrations used and the results obtained. Rubidium proved to be a good substitute while neither thorium nor uranium were capable of taking the place of potassium. As soon as potassium was added, growth started in those cultures which heretofore had shown no growth.

TABLE 4

Saccharomyces cerevisiae with various potassium substitutes (cells per cubic centimeter)

	TO 100 CC. OF THE K-FREE MEDIUM WAS ADDED				
	0	10 mgm. KCl	15 mgm. RbCl	5 mgm. $ThNO_3$	2.5 mgm. $UO_2(NO_3)_2$
Start	54,000	58,000	(56,000)	(56,000)	(56,000)
2 days	87,000	2,025,000	640,000	72,000	72,500
3 days	77,500	4,450,000	1,035,000	76,000	71,500
4 days	84,000	5,200,000	2,400,000	83,000	85,000
6 days	120,000	8,500,000	2,590,000	77,000	92,000
Added to each culture 100 mgm. KCl (= 0.1 per cent)					
8 days	2,400,000	4,500,000	5,000,000	2,400,000	3,000,000

TABLE 5

Growth of yeast in potassium-uranium mixtures (cells per cubic centimeter)

	TO 100 CC. OF THE POTASSIUM-FREE MEDIUM WAS ADDED					
KCl $UO_2(NO_3)_2 \cdot 6H_2O$	0 2.5 mgm.	0 10 mgm.	10 mgm. 0	10 mgm. 2.5 mgm.	10 mgm. 5 mgm.	10 mgm. 10 mgm.
Start	8,700	8,700	8,700	8,700	8,700	8,700
After 2 days	13,300	13,400	1,400,000	2,500,000	4,300,000	700,000
After 4 days	4,900	850	2,500,000	3,600,000	10,600,000	2,100,000

The purpose of the third series was to try whether the antagonism claimed by Zwaardemaker between K and Rb on one side and Th, U, and Ra on the other side would become evident in yeast growth. Media and test organisms were the same as in the preceding experiment. To the K-free solution were added either K or U salts, or a mixture of both. Table 5 shows that neither large nor small doses of U can take the place of K, but

there is no evidence of antagonism. On the contrary, a distinct stimulative effect by 5 mgm. U O_2 $(NO_3)_2 \cdot 6H_2O$ can be noticed. Such stimulating effects have been observed with bacteria by Stoklasa and Penkava (1932) and are ascribed to the β and γ radiation of uranium. However, their concentration showing maximum stimulation (12.5×10^{-6} gram atoms of U per liter = 0.63 mgm. uranyl-nitrate in 100 cc.) is only one-tenth of the amount found most efficient in yeast.

Bayliss (1924) suggests that the rôle of potassium might be related to the great mobility of the K ion. "In the formation of a Helmholtz double layer, potassium will out-distance other cations and, therefore, tend to be in excess in the positively charged side

TABLE 6

Properties of the alkali metals

ELEMENT	ATOMIC WEIGHT	ATOMIC NUMBER	ION-MOBILITY	RADIATION
Lithium	6.94	3	33.4	0
Sodium	22.997	11	43.5	0
Potassium	39.10	19	64.6	β
Rubidium	85.44	37	67.5	β
Cesium	132.81	55	68	0
Radium	225.97	88	58	$\alpha + \beta$
Thorium	232.12	90		α
Uranium	238.14	92		$\alpha + \beta$

of the layer." If we compare the properties of the elements of the same chemical group (table 6) we see that rubidium is the only element with β-radiation and a similar transference number. Li and Na have much smaller ion mobilities and are not radio-active; Cs has a similar mobility, but is not radio-active. Ra, Th, U have different mobilities, but being divalent elements of a different group, could hardly be expected to be substitutes for any function other than supply of β-particles.

It seems safe to conclude from this comparison that the rôle of potassium in those organisms which need it, is not limited to its β-radiation, but that this radiation may be part of its function in the cell; otherwise, cesium might be as good a substitute as rubidium.

SUMMARY

Aspergillus niger, two species of *Saccharomyces*, various species of *Mycobacterium* and all Gram-positive bacteria so far investigated require potassium for their growth. The only element capable of replacing K in yeasts, mycobacteria or aerobic spore-formers is rubidium though the crops are smaller.

The evidence regarding substitution by cesium is contradictory in *Aspergillus*, negative in the other organisms. Lithium or sodium cannot replace potassium, nor can the bivalent radio-active elements do this. Since rubidium is the only substitute, it seems that the radio-activity of potassium is an essential factor in its rôle, but not the only factor.

The Gram-negative bacteria investigated so far, with the exception of *Rhizobium*, can grow without potassium. At least, they develop to large numbers in media made from analyzed C.P. chemicals in glass containers, and addition of potassium salts does not greatly increase the crop. Gram-positive bacteria develop in such media only after addition of potassium salt.

REFERENCES

BAYLISS, W. M.: Principles of General Physiology, 1924, p. 217.
BENECKE, W.: Pringsh. Jahrb. f. wissenschaftl. Botanik, 1895, **28**, 487.
BESANCON, PHILBERT AND BOUDIN: Bull. Institut Pasteur, 1913, **11**, 578.
BOISSEVAIN, C. H.: Jour. Infect. Diseases, 1928, **43**, 194.
CACHE, A.: Zent. Bakteriol., I Abt., Orig., 1906, **40**, 255.
FRED, E. B., BALDWIN, I. L., AND MCCOY, E.: Root Nodule Bacteria and Leguminous Plants. Madison, 1932, p. 110.
FROUIN, A., AND GUILLAUMIE, M.: Compt. Rend. Soc. Biol., 1926, **94**, 1115.
GERLACH, M., AND VOGEL, J.: Zent. Bakteriol., II Abt., 1901, **7**, 109.
KOCH, G. P.: Abstr. Bacteriol., 1918, **2**, 3.
LOCKEMANN, G.: Zent. Bakteriol., I Abt., Orig., 1919, **83**, 420.
LÖWENSTEIN, S.: Zent. Bakteriol., I Abt., Orig., 1913, **68**, 591.
NÄGELI, C. VON: Bot. Zentralbl., 1882, **12**, 345.
RAULIN, J.: Compt. Rend. Acad. Sciences, 1870, **70**, 634.
SAUTON, B.: Compt. Rend. Acad. Sciences, 1912, **155**, 860 and 1181.
STOKLASA, J., AND PENKAVA, J. Biologie des Radiums und Uraniums. Berlin, Parey, 1932, 262.
WAKSMAN, S. A.: Principles of Soil Microbiology. Baltimore, 1932, p. 506.
ZUNZ, E., AND GYÖRGI, P.: Jour. Bacteriol., 1916, **1**, 627.
ZWAARDEMAKER, H.: Ergebnisse der Physiologie, 1921, **19**, 326.

THE SEROLOGIC AGGLUTINATION OF THE OBLIGATE ANAEROBES CLOSTRIDIUM PARAPUTRIFICUM (BIENSTOCK) AND CLOSTRIDIUM CAPITOVALIS (SNYDER AND HALL)

MARSHALL L. SNYDER

From the Department of Bacteriology and Public Health and the Child Research Council, University of Colorado School of Medicine and Hospitals, Denver, Colorado

Received for publication May 4, 1936

Early workers, LeClainche and Vallee (1900), Bachmann (1904), Rocchi (1911), Markoff (1911), Meyer (1915) and others, differentiated *Clostridium oedematismaligni* (Koch), Ghon and Sachs bacillus (*Vibrion septique*), and the "Rauschbrandbacillus" (*Clostridium chauvei*) by agglutination. On the other hand, Zeissler (1919) and Heller (1920) condemned the agglutination test for sporulating anaerobes because of the occurrence of spontaneous agglutination and cross-reactions of certain sera with unrelated species.

More recently, however, several investigators have used serologic agglutination tests in the identification of anaerobic bacilli without encountering either spontaneous agglutination or non-specific cross-agglutination, as witness the work of Goss, Barbarin and Haines (1921) with *Cl. chauvei* and the *Vibrion Septique*, Starin and Dack (1923) with *Clostridium sporogenes*, *Clostridium botulinum*, and *Clostridium putrificum*, Hall and Stark (1923) with *Cl. sporogenes*, Hall and Scott (1931) with *Clostridium Sordellii*, Duffett (1935) with *Clostridium carnis*, and McCoy and McClung (1935) with *Clostridium acetobutylicum*.

Agglutination methods, moreover, have demonstrated serological groups in several anaerobic species, particularly in *Clostridium tetani* by Tulloch (1919), Bauer (1924), Fildes (1925), and Coleman and Gunnison (1928) and in *Cl. botulinum* by Schoenholz and Meyer (1923).

In the light of these facts it was thought of interest to report the agglutination reactions which not only differentiated sharply *Clostridium paraputrificum* from *Clostridium capitovalis*, but also established serological subgroups in *Cl. paraputrificum*. Acid agglutination was encountered and especially studied because of its rôle in giving false reactions in these anaerobic agglutination tests.

The cultural characteristics separating these two non-pathogenic, terminal, oval-spored anaerobes isolated from intestinal contents and postmortem material were reported by Hall and Snyder (1934) and Snyder and Hall (1935). They showed that *Cl. paraputrificum* was obligately saccharolytic and probably identical with Escherich's "Kopfchenbakterien" (1886) and Rodella's Bacillus III (1902), but had been adequately described and named by Bienstock (1906). A careful search of the literature, however, failed to show any organism identical with the one named *Cl. capitovalis*, which differed from *Cl. paraputrificum* in being mildly proteolytic.

METHODS

Agglutinating sera of a high titer were prepared in rabbits by a series of nine or more subcutaneous injections of 2.0 cc. of forty-eight-hour glucose broth cultures at two to five-day intervals. Prior to the first inoculations no normal agglutinins were demonstrated in the rabbits. When a series of inoculations was finished, ten days or more were allowed to lapse before the rabbit was bled from the marginal ear vein for a preliminary titration of the serum. If this titration was found to be satisfactory, larger quantities of serum were obtained by cardiac bleeding.

Forty-eight-hour glucose broth cultures in Hall's constricted tubes were used for the agglutination tests. The cultures were filtered through cotton and diluted to an opacity of 300 on the turbidity scale recommended in "Standard Methods of Water Analysis" (1923). Titrations of the various sera were carried out by diluting the sera 1:10 and doubling the dilutions up to 1:2560. The addition of an equivalent amount of bacterial culture then

gave serum dilutions from 1:20 to 1:5120. The mixtures were incubated at 37°C. Because of the apparently slow agglutination the first tests were recorded at 12 hours, but it was found later that seven hours were sufficient for complete flocculation.

DATA

For the preliminary analysis of eighteen strains of *Cl. paraputrificum* and seven strains of *Cl. capitovalis*, a fecal strain of *Cl. paraputrificum*, no. 4453, was selected. After a satisfactory agglutinating serum was prepared, tests were carried out against the other strains according to the technique outlined. The results are given in table 1.

Table 1 shows that all strains of *Cl. paraputrificum* were partially or completely agglutinated in a serum dilution of 1:40. Of these strains, however, nos. 1538, 1545, 3698, and 4388 were flocculated as strongly as the homologous culture, while strains 3572 and 4065were even more strongly flocculated, indicating an antigenic subdivision in the species *Cl. paraputrificum*. The *Cl. capitovalis* strains, except no. 3904 which was not agglutinated, showed traces of agglutination in all tubes including the saline controls and complete flocculation in a 1:320 dilution with strain 674. These positive reactions could not be explained at this time.

The presence of serological groups in *Cl. paraputrificum* was further shown by preparing an agglutinating serum against a strain only slightly agglutinated by antiserum 4453; strain 4042 was selected. This serum was tested against the eighteen *Cl. paraputrificum* and seven *Cl. capitovalis* strains. It was found that strains 3978, 3941, 3909, and 4351 which were not markedly agglutinated by antiserum 4453 were flocculated as strongly as the control, or even more strongly, while strains 1538, 1545, 3698, 3572, 4388, and 4065 brought down strongly by antiserum 4453 were weakly agglutinated by antiserum 4042. This confirmed the presence of at least one antigenic subdivision in the species *Cl. paraputrificum*. Cross-agglutination with *Cl. capitovalis* strains showed partial flocculation in all tubes including the saline con-

TABLE 1

Cross-agglutination tests of Cl. paraputrificum and Cl. capitovalis strains with Cl. paraputrificum antiserum 4453

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+++	+++	++	−	−	−	−	−	−	−
	491	++++	++++	++++	+++	++	−	−	−	−	−
	1538	++++	++++	++++	+++	+++	++	+	−	−	−
	1545	++++	++++	++++	++++	++++	+++	++	+	−	−
	3572	++++	++++	++++	++++	++++	++++	++++	++++	+++	−
	3638	++++	++++	+++	++	−	−	−	−	−	−
	3698	++++	++++	+++	+++	++	−	−	−	−	−
	3941	+++	++	+	−	−	−	−	−	−	−
	3953	++++	++	−	−	−	−	−	−	−	−
	4042	+++	++	−	−	−	−	−	−	−	−
	3909	++++	++	+	−	−	−	−	−	−	−
	3945	+++	+++	++	+	−	−	−	−	−	−
	4013	++++	+++	++	+	−	−	−	−	−	−
	4104	++++	++++	+++	+	−	−	−	−	−	−
	4351	++	++	++	−	−	−	−	−	−	−
	4388	+++	+++	+++	++	+	+	+	+	−	−
	4453*	++++	++++	++++	++++	++++	+++	+++	+	+	−
	4065	++++	++++	+++	+++	++	++	++	+	+	−
Cl. capitovalis	137	+	+	+	+	+	+	+	+	+	+
	2076	+	+	+	+	+	+	+	+	+	+
	3543	+	+	+	+	+	+	+	+	+	+
	3904	−	−	−	−	−	−	−	−	−	−
	4236	+	+	+	+	+	+	+	+	+	+
	613	+	+	+	+	+	+	+	+	+	+
	674	+	+	+	+	++++	+	+	+	+	+

* Homologous strain.

trols of strains 4236 and 613, but not with the remaining five strains. These positive results could not be explained at the time of reading.

While these tests were being made, an agglutinating serum was prepared against a strain of *Cl. capitovalis*, no. 137. Cross-agglutination tests were carried out with this and the remaining strains of *Cl. paraputrificum* and *Cl. capitovalis*. All strains of *Cl. capitovalis* agglutinated to nearly the same degree as the control. The reactions of the *Cl. paraputrificum* strains, however, were of considerable interest because all strains showed some agglutination in a 1:20 serum dilution, many in a 1:40 dilution, and a few at 1:80. The unusual thing appeared to be the sharp end-point rather than the tapering off, ordinarily encountered in specific agglutination. These agglutinations were suspected to be the results of the action of the acid produced in the glucose broth by the vigorous fermentation of this sugar by *Cl. paraputrificum*, and corresponded to the type of acid agglutination described by Michaelis (1911) and others. Colorimetric tests with brom-cresol-green showed all *Cl. paraputrificum* strains produced a terminal acidity in glucose broth of pH 4.4 to 4.8, while *Cl. capitovalis* strains were slightly higher at pH 4.9 to 5.1.

The accuracy of this hypothesis was tested by adding N/10 NaOH to the glucose broth cultures until neutrality was approximated as tested by brom-thymol-blue on aliquot samples. These neutralized cultures were then tested against antiserum 137 in parallel with a series of acid cultures as controls. The acid series gave essentially the same results as before. The results with the neutralized cultures are listed in table 2.

Table 2 shows that while neutralization lowered the titer limits of the homologous and related *Cl. capitovalis* strains, it almost eliminated the pseudo-reactions of the *Cl. paraputrificum* strains as previously encountered. Despite the apparent specificity of these results, a question arose over the possible influence of the salts formed in the neutralization process.

To avoid this factor the cultures were grown in neutral peptone infusion broth in Hall's constricted tubes. While growth was not as heavy as in glucose broth, it was usually sufficient to carry out

TABLE 2

Cross-agglutination tests of Cl. paraputrificum and Cl. capitovalis strains with Cl. capitovalis antiserum 157

Forty-eight-hour glucose broth cultures neutralized with N/10 NaOH

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+	+	−	−	−	−	−	−	−	−
	491	−	−	−	−	−	−	−	−	−	−
	1538	−	−	−	−	−	−	−	−	−	−
	1535	−	−	−	−	−	−	−	−	−	−
	3572	−	−	−	−	−	−	−	−	−	−
	3638	−	−	−	−	−	−	−	−	−	−
	3698	+	+	−	−	−	−	−	−	−	−
	3941	−	−	−	−	−	−	−	−	−	−
	3953	−	−	−	−	−	−	−	−	−	−
	4042	−	−	−	−	−	−	−	−	−	−
	3909	−	−	−	−	−	−	−	−	−	−
	3945	−	−	−	−	−	−	−	−	−	−
	4013	−	−	−	−	−	−	−	−	−	−
	4104	−	−	−	−	−	−	−	−	−	−
	4351	−	−	−	−	−	−	−	−	−	−
	4388	−	−	−	−	−	−	−	−	−	−
	4453	−	−	−	−	−	−	−	−	−	−
	4065	−	−	−	−	−	−	−	−	−	−
Cl. capitovalis	137*	++++	++++	++++	++++	++++	++	+	−	−	−
	2076	++++	++++	++++	++++	+++	++	+	−	−	−
	3543	++++	++++	++++	+++	++	++	−	−	−	−
	3904	++++	++++	++++	++++	++	+	−	−	−	−
	4236	++++	++++	++++	++++	++++	++	++	−	−	−
	613	++++	++++	++++	++++	+++	++	+	−	−	−
	674	++++	++++	++++	+++	+++	++	+	−	−	−

* Homologous strain.

TABLE 3

Cross-agglutination tests with Cl. paraputrificum and Cl. capitovalis strains with Cl. paraputrificum antiserum 4453

Forty-eight-hour peptone infusion broth cultures used

	STRAIN NUMBER	SERUM DILUTIONS									CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Cl. paraputrificum	3978	+++	++	+	–	–	–	–	–	–	–
	491	++++	+++	++	–	–	–	–	–	–	–
	1538	++++	++++	++	–	–	–	–	–	–	–
	1545	+++	++	–	–	–	–	–	–	–	–
	3572	++++	++++	++++	++++	++++	++++	++++	++	–	–
	3638	+++	++	–	–	–	–	–	–	–	–
	3698	++++	++++	++++	++++	++++	++++	–++++	+++	++	–
	3941	++	–	–	–	–	–	––	–	–	–
	3953	+++	++	–	–	–	–	––	–	–	–
	4042	++++	++	–	–	–	–	–	–	–	–
	3909	++++	++	–	–	–	–	–	–	–	–
	3945	++++	++++	–	–	–	–	–	–	–	–
	4013	+++	++	–	–	–	–	–	–	–	–
	4104	+++	++	–	–	–	–	–	–	–	–
	4351	++++	++	–	–	–	–	–	–	–	–
	4388	++++	++++	++	–	–	–	–	–	–	–
	4453*	++++	++++	++++	++++	+++	++	–	–	–	–
	4065	+++	++	++	–	–	–	–	–	–	–
Cl. capitovalis	137	–	–	–	–	–	–	–	–	–	–
	2076	–	–	–	–	–	–	–	–	–	–
	3543	–	–	–	–	–	–	–	–	–	–
	3904	–	–	–	–	–	–	–	–	–	–
	4236	–	–	–	–	–	–	–	–	–	–
	613	–	–	–	–	–	–	–	–	–	–
	674	–	–	–	–	–	–	–	–	–	–

* Homologous strain.

the agglutination tests. At the end of forty-eight hours the hydrogen-ion concentration of the peptone infusion broth cultures was not appreciably changed. Cross-agglutination tests were then carried out with antiserum 137. A control series of acid cultures was also carried out with results similar to those previously described. Even sharper differentiation between the strains of *Cl. capitovalis* and *Cl. paraputrificum* was obtained with the use of peptone infusion broth cultures. The apparent antigenic homogeneity of *Cl. capitovalis* was retained.

The process was repeated with *Cl. paraputrificum* agglutination serum 4453. Control acid cultures gave results similar to those in table 1 with *Cl. paraputrificum* strains, and partial agglutination in the 1:20 and 1:40 serum dilutions with *Cl. capitovalis* strains 137, 2076, 4236, 613, and 674. The results with the peptone infusion broth cultures are listed in table 3.

The data in table 3 confirm the sharp demarcation serologically between the two species when the disturbing acid factor was eliminated. The results of these agglutination tests with *Cl. paraputrificum* demonstrate again the existence of antigenic subdivisions in the species as shown in table 1.

DISCUSSION

The evidence submitted showed that the agglutination test separated *Cl. paraputrificum* and *Cl. capitovalis* as distinct species which confirmed the previously reported cultural studies on these two morphologically similar anaerobes. The larger number of *Cl. paraputrificum* strains afforded a better opportunity to establish antigenic subdivisions in the species than the smaller number of *Cl. capitovalis* strains, but no attempt has yet been made to confirm by receptor analysis the antigenic heterogeneity in *Cl. paraputrificum* as established by the agglutination tests. Cross-agglutination tests with other non-pathogenic, terminal, oval-spored anaerobes, *Clostridium cochlearium*, *Clostridium tetanomorphum*, *Clostridium caloritolerans*, and *Clostridium putrificum*, were all negative.

This study served to determine roughly the terminal acidity above which broth cultures should not be used in agglutination

tests. Colorimetrically the glucose broth cultures of *Cl. paraputrificum* strains were under pH 4.8 while *Cl. capitovalis* strains were usually slightly above. Thus the critical point for the non-specific acid agglutination of broth cultures of these two species was apparently close to pH 4.8. Subsequent tests with broth suspensions of *Cl. paraputrificum*, *Cl. capitovalis*, and other anaerobic species showed that the broth rather than the organisms determined this type of acid agglutination.

It is obvious that the use of glucose broth cultures in agglutination tests with sporulating anaerobes may lead to spurious results, particularly if the hydrogen-ion concentration is overlooked and increases to give pH values below 5.0. This difficulty may be overcome in two ways; (1) by using neutralized glucose broth cultures, and (2) by growth of cultures in media containing so little carbohydrate that the pH is not significantly altered.

SUMMARY

Clostridium paraputrificum and Clostridium capitovalis were shown serologically to be distinct species.

Non-specific acid agglutinations were shown to occur when glucose broth cultures of these anaerobes with terminal acidity below pH 5.0 were used in agglutination tests.

REFERENCES

American Public Health Association. Standard Methods of Water Analysis, 1923.
Bachmann, E. 1911 Centralbl. f. Bakt., Orig. I, **37**, 221.
Bauer, J. H. 1924 Proc. Soc. Exper. Biol. and Med., **21**, 265.
Bienstock, B. 1906 Ann. Inst. Past., **20**, 407.
Coleman, G., and Gunnison, J. B. 1928 Jour. Inf. Dis., **43**, 184.
Duffett, N. D. 1935 Jour. Bact., **29**, 573.
Escherich, T. 1886 Dissertation, Stuttgart.
Fildes, P. 1925 Brit. Jour. Exper. Path., **6**, 91.
Goss, L. W., Barbarin, R. E., and Haines, A. W. 1921 Jour. Inf. Dis., **29**, 615.
Hall, I. C., and Scott, A. L. 1931 Jour. Bact., **22**, 375.
Hall, I. C., and Snyder, M. L. 1934 Jour. Bact., **28**, 181.
Hall, I. C., and Stark, N. 1923 Jour. Inf. Dis., **33**, 240.
Heller, H. H. 1920 Jour. Inf. Dis., **27**, 385.
LeClainche, E., and Vallee, H. 1900 Ann. Inst. Past., **14**, 513.
Markoff, W. N. 1911 Centralbl. f. Bakt., Orig. I, **60**, 188.
McCoy, E., and McClung, L. S. 1935 Jour. Inf. Dis., **56**, 333.
Meyer, K. F. 1915 Jour. Inf. Dis., **17**, 458.

MICHAELIS, L. 1911 Deut. med. Woch., **37**, 969.
ROCCHI, G. 1911 Centralbl. f. Bakt., Orig. I, **60**, 579.
RODELLA, A. 1902 Ztschr. f. Hyg., **39**, 201.
SCHOENHOLZ, P., AND MEYER, K. F. 1923 Jour. Inf. Dis., **32**, 417.
SNYDER, M. L., AND HALL, I. C. 1935 Centralbl. f. Bakt., Orig. I, **135**, 290.
STARIN, W. A., AND DACK, G. M. 1923 Jour. Inf. Dis., **33**, 169.
TULLOCH, W. J. 1919 Jour. Hyg., **18**, 103.
ZEISSLER, J. 1919 Berl. kl. Woch., **56**, 107.

MECHANISM OF THE NON-SPECIFIC SERUM AGGLUTINATION OF THE OBLIGATE ANAEROBES CLOSTRIDIUM PARAPUTRIFICUM AND CLOSTRIDIUM CAPITOVALIS

MARSHALL L. SNYDER

From the Department of Bacteriology and Public Health and the Child Research Council, University of Colorado School of Medicine and Hospitals, Denver, Colorado

Received for publication May 4, 1936

Non-specific reactions in agglutination tests have long provided trouble for the bacteriologist. One of the chief offenders has been spontaneous agglutination. The pseudo-reactions obtained in the serologic agglutination of the obligate anaerobes *Clostridium paraputrificum* (1934) and *Clostridium capitovalis* (1935), however, were not considered as due to spontaneous agglutination, but rather to the acid produced in the glucose broth cultures used. When the acid was eliminated it was easy to differentiate these species on the basis of agglutination.

At first it was thought that acid alone was responsible for the cross-agglutinations occurring when glucose broth cultures of these species were tested against heterologous serums. It was found, however, that a constituent of the broth was also a contributing factor. The relation of these serum-broth-acid reactions to the acid agglutinations described by Michaelis (1911) was also studied.

I. THE pH VALUES OF NON-SPECIFIC AGGLUTINATION WITH BROTH CULTURES OF CL. PARAPUTRIFICUM

The pH values at which non-specific agglutination occurred with broth cultures of *Cl. paraputrificum*, strain 4453, was roughly determined by using buffer solutions of pH 7.0, 6.0, 5.0, and 4.0 approximately. These were made according to McIlvaine's

tables (1921). Normal rabbit serum was then diluted from 1:10 to 1:2560 in each buffer solution in order to maintain the customary 2.0 cc. volume of the macroscopic agglutination test. These serum-buffer mixtures were allowed to stand at least one hour. No changes were observed in this time or after three hours incubation. Because *Cl. paraputrificum* at times failed to grow in peptone meat infusion broth heavily enough to provide satisfactory suspensions, the organisms were grown in glucose broth for forty-eight hours, twice centrifugalized and washed in distilled water, and re-suspended in peptone meat infusion broth. These suspensions were filtered through cotton and then added in equal quantities to the serum-buffer mixtures. Controls of the peptone meat infusion suspensions and of each buffer without serum were included in each test. At the same time a parallel series of glucose broth cultures was tested. The mixtures were incubated at 37°C. for three hours before the reactions were recorded and the hydrogen-ion concentrations determined colorimetrically. Controls of the bacterial suspensions and normal serum diluted in physiological saline were also tested. The two series are compared in table 1.

Table 1 shows that agglutination occurred in both series between pH 4.6 to 5.2. No flocculation occurred with the peptone infusion broth suspension in serum-saline at the pH of 6.8, but partial agglutination was found up to 1:80 in the serum-saline and glucose broth culture mixtures, the pH values of which were approximately 5.1. These reactions indicated that in a narrow pH zone serum in concentrations greater than 1:80 aided flocculation because any marked increase in hydrogen-ion concentration gave more or less complete agglutination in all tubes including the controls with no serum.

To determine as closely as possible the critical value of the reaction, potentiometric measurements were made with a glass electrode.[1] Cultures of *Cl. paraputrificum* were grown for forty-eight hours at 37°C. in glucose broth under "vas-par," twice centrifugalized and washed in distilled water, and resuspended in

[1] The author is indebted to Dr. Robert M. Hill of the Department of Biochemistry for these measurements.

TABLE 1

Zone of hydrogen-ion concentration giving non-specific agglutination of broth cultures of Cl. paraputrificum in the presence of normal serum

pH RANGE	SERUM DILUTIONS									NO SERUM
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
A. Peptone meat infusion suspensions										
7.0	–	–	–	–	–	–	–	–	–	–
6.1	–	–	–	–	–	–	–	–	–	–
5.2	–	–	–	–	–	–	–	–	–	–
4.4–4.6	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
6.8*	–	–	–	–	–	–	–	–	–	–
B. Glucose broth cultures										
7.1	–	–	–	–	–	–	–	–	–	–
6.2	–	–	–	–	–	–	–	–	–	–
5.2	–	–	–	–	–	–	–	–	–	–
4.4–4.6	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4.9–5.1*	+++	++	++	–	–	–	–	–	–	–

* Saline controls.

TABLE 2

Agglutination of peptone broth suspensions of Cl. paraputrificum in serum-acid buffers, the pH of the mixtures being tested potentiometrically

Agglutinations read at three hours

pH RANGE	SERUM DILUTIONS						NO SERUM	pH
	1:20	1:40	1:80	1:160	1:320	1:3200		
6.92–6.96	–	–	–	–	–	–	–	6.63
5.61–5.63	–	–	–	–	–	–	–	5.61
5.09–5.10	–	–	–	–	–	–	–	5.00
4.97–5.00	+++	++	–	–	–	–	–	4.80
4.87	++++	++	++	++	–	–	–	4.80
4.73–4.80	++++	++++	++++	+++	+++	+++	+++	4.67
4.49–4.52	++++	++++	++++	++++	++++	++++	++++	4.42
4.20–4.40	++++	++++	++++	++++	++++	++++	++++	4.35
4.85–5.00*	+++	+++	++	–	–	–	–	4.83

*Saline control.

neutral peptone infusion broth. Normal rabbit serum was diluted in buffer solutions of pH 7.05, 6.06, 5.20, 5.04, 4.85, 4.53, 4.2, and 4.03, respectively. Instead of the complete series of nine dilutions of serum only the first five, namely, 1:20, 1:40, 1:80, 1:160, and 1:320 were tested. A sixth dilution of 1:3200 was included to test for the effect of dilute serum concentration. Controls included buffers without serum and a series of normal serum diluted in physiological saline with a glucose broth culture for the agglutinating suspension. The volumes for these tests were 5.0 cc. serum dilutions and 5.0 cc. of the bacterial suspensions. After mixing and incubating for a half hour, the potentials were measured and the corresponding pH values determined. Part of the mixture in each tube was used for the pH determination and the remainder for agglutination titer. The results are listed in table 2.

Table 2 shows that in a mixture of normal serum and *Cl. paraputrificum* in peptone infusion broth adjusted to different degrees of acidity by buffer solutions the critical pH value for flocculation was between pH 5.0 to 5.10. Above pH 5.10 flocculation did not take place in any tube of the series. In a narrow zone below this value, pH 4.80 to 5.10, only tubes containing the highest concentrations of serum were affected, but below pH 4.80 all tubes showed more or less equal flocculation. The control of serum in physiological saline solution to which glucose broth culture was added showed partial agglutination in serum dilutions up to 1:80.

The complete or nearly complete agglutination of broth suspensions of *Cl. paraputrificum* below pH 4.8 compared with a preliminary observation that watery or saline suspensions of *Cl. paraputrificum* were stable at pH 4.0 indicated that acid alone was not responsible for the non-specific reactions encountered. Therefore, the following experiments were designed to point out other possible factors.

II. OTHER FACTORS RESPONSIBLE FOR NON-SPECIFIC AGGLUTINATION OF BROTH CULTURES OF CL. PARAPUTRIFICUM

Normal serum was diluted as before in known acid buffer solutions and equivalent amounts of the following added: peptone

TABLE 3

Agglutination of Cl. paraputrificum in various media by known acid concentrations

	SERUM DILUTIONS									NO SERUM	pH RANGE
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120		
Peptone meat infusion	–	–	–	–	–	–	–	–	–	–	5.2
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Meat infusion	–	–	–	–	–	–	–	–	–	–	5.2
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Peptone water	–	–	–	–	–	–	–	–	–	–	4.7–4.9
	–	–	–	–	–	–	–	–	–	–	4.4–4.6
Uninoculated meat infusion	+++	++	–	–	–	–	–	–	–	–	4.9–5.0
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	4.4–4.6
Saline	–	–	–	–	–	–	–	–	–	–	5.2
	–	–	–	–	–	–	–	–	–	–	4.4–4.6
Water	–	–	–	–	–	–	–	–	–	–	5.2
	–	–	–	–	–	–	–	–	–	–	4.4–4.6

TABLE 4

Acid agglutinations of sporulating anaerobes

	ACID CONCENTRATIONS EXPRESSED IN pH								
	5.70	5.10	4.80	4.33	3.84	3.45	3.01	2.60	2.20
Cl. Welchii	–	–	++	–	–	–	–	–	–
Cl. Novyi	–	++	+++	++++	++++	++	–	–	–
Cl. septicum	–	–	–	–	–	++	++++	++++	++++
Cl. tetani	–	–	++	+++	–	–	–	–	–
Cl. sporogenes	+++	++++	+++	++++	++++	++++	++++	++++	++++
Cl. botulinum B	–	–	–	–	–	–	–	++++	++++
Cl. bifermentans	–	–	–	–	–	–	–	–	–
Cl. difficilis	–	–	–	–	–	–	–	++++	++++
Cl. paraputrificum	–	–	–	–	–	–	++	++	++
Cl. capitovalis	–	–	+++	+++	++++	++++	++++	+++	++

infusion broth, meat infusion broth, peptone water, saline, and distilled water suspensions of *Cl. paraputrificum*. A control series with uninoculated meat infusion broth was also tested. The final hydrogen-ion concentrations of the mixtures were tested colorimetrically. The significant results obtained with each suspension are listed in table 3.

Table 3 shows that the meat infusion and peptone meat infusion broth suspensions of *Cl. paraputrificum* flocculated in the pH range 4.4 to 4.6, but not at 5.2. The uninoculated meat infusion broth also flocculated in the same range and partially in the serum dilutions 1:20 and 1:40 at 4.9 to 5.0. Suspensions of *Cl. paraputrificum* in water, saline solution, and peptone solution were stable in all tubes. These results suggested that the acid precipitated some substance of the meat infusion, which in turn brought down the bacteria to give the non-specific reactions. Contrary to the observation of de Kruif (1921) the presence of peptone apparently did not extend the zone of acid agglutination. The stability of water and saline suspensions of *Cl. paraputrificum* in the presence of the serum-buffer mixtures indicated that the bacteria were not directly agglutinated by the acid, but it remained to test the influence of acid on *Cl. paraputrificum* without the presence of broth or serum.

III. ACID AGGLUTINATION OF CL. PARAPUTRIFICUM AND OTHER SPORULATING ANAEROBES

Acid agglutination was first utilized by Michaelis (1911) as a possible means of identifying bacteria by their flocculation at the isoelectric points of their specific proteins. He was thus able to distinguish *Eberthella typhosa*, *Salmonella paratyphi* and *Salmonella Schottmuelleri*. His pupil Beniasch (1912) recorded the isoelectric point of a large number of aerobic species of bacteria. Gieszczykiewicz (1916) established acid agglutination values for several of the sporulating anaerobes as follows: *Clostridium tetani*, pH 4.16; *Clostridium botulinum* and *Clostridium chauvei*, pH 3.55; and *Clostridium butyricum*, pH 2.96. The succeeding controversy over the value of this test as a diagnostic procedure was ably reviewed by Fischer (1928), who investigated the acid agglutina-

tion of the hemophilic organisms. He arrived at the same conclusion as many others; namely, the test might apply to some species, but any positive value was limited by the overlapping zones of agglutination of many species.

For the acid agglutinations in the following tests *Cl. paraputrificum* was grown in glucose broth for forty-eight hours, after which it was centrifugalized, then twice washed and centrifugalized, and re-suspended in distilled water. The suspensions were filtered through cotton before using. To 3.0 cc. volumes of this suspension placed in each of nine tubes 1.0 cc. of each known buffer solution was added respectively. The tubes were incubated overnight at 37°C. The same technique was used for the other sporulating anaerobes tested, which were *Clostridium Welchii*, *Clostridium novyi*, *Cl. septicum*, *Cl. tetani*, *Clostridium sporogenes*, *Clostridium bifermentans*, *Cl. difficilis*, and *Cl. capitovalis*. The results obtained are given in table 4.

Table 4 shows that with *Cl. Welchii* only was agglutination limited to a single tube, namely pH 4.80. The others had zones of agglutination ranging from two tubes with *Cl. tetani* and *Cl. difficilis* to nearly complete agglutination in all nine tubes with *Cl. sporogenes*. *Cl. bifermentans* was not agglutinated in any hydrogen-ion concentration studied. The zone of partial agglutination of *Cl. paraputrificum* extended from pH 3.01 to 2.20.

DISCUSSION

Broth cultures of sporulating anaerobes for agglutination tests were used by Robertson (1916), Fildes (1925), and others. Fildes, however, worked with *Cl. tetani* which is non-saccharolytic and cannot give acid agglutination. Hall and Stark (1923) found glucose broth cultures of *Cl. sporogenes* satisfactory for agglutination purposes; later, Hall and Scott (1931) used similar antigens in the serologic study of *Clostridium Sordellii*. Although these two species ferment glucose vigorously, the failure of these workers to encounter non-specific agglutination suggests that the hydrogen-ion concentration of their glucose broth cultures was less than that needed for the acid agglutination of broth cultures of these species.

The fundamental differences between the acid agglutination of Michaelis and the agglutination in the acid-broth-serum-bacteria complex in this study were soon noted. Michaelis used bacteria suspended in distilled water and added acid buffer solutions ranging from pH 3.5 to 5.0. In the present experiments, however, the set-up was that of an ordinary agglutination test; that is, the serum was diluted in either physiological saline solution or buffer solutions from 1:10 to 1:2560 to which were added equivalent amounts of glucose or broth suspensions, doubling the serum dilutions. A control tube contained no serum. Hence, serum and broth with its various components, normal and those of bacterial metabolism, were present in contrast to their absence in the so-called acid agglutination test.

Although the experiments are confined to anaerobic species they illustrate by the overlapping of acid agglutination zones the limitations of Michaelis' test in separating bacterial species. It may be noted also that the agglutination zone, pH 2.60 to 2.20 for *Cl. botulinum* does not coincide with the optimum, pH 3.55, given for this species by Gieszczykiewicz. The optimum for *Cl. tetani* in these results may be at some point between pH 4.33 to 3.84, which would approximate the value, pH 4.16, given by Gieszczykiewicz for this species. The acid agglutination of *Cl. paraputrificum* was far below the acid values tested in the broth series, which further suggests the action is due to the flocculation of some other substance than the bacterial cells to give the non-specific results encountered with glucose broth cultures of *Cl. paraputrificum*.

The influence of serum on acid agglutination was discussed by Michaelis (1917) who claimed that *Escherichia coli*, which was not susceptible to acid agglutination, was flocculated in the presence of normal serum, while *Shigella dysenteriae* was not. Krumwiede and Pratt (1912) showed that typhoid bacteria sensitized with immune serum beyond the titer limits so that no agglutination occurred were agglutinated in lower acid concentrations than unsensitized bacteria. Gieszczykiewicz demonstrated that normal serum in a 1:100 concentration almost eliminated acid agglutination, whereas a 1:1000 concentration exerted no interference.

He also confirmed the observation of Krumwiede and Pratt. In the present experiments it was apparent that serum played little rôle. Only in the narrow zone of pH 4.8 to 5.10 was the combination of broth, bacteria, and normal serum in concentrations greater than 1:80 more sensitive to the action of acid. Outside of these values either complete stability or flocculation was usually noted.

Thus it was apparent that the agglutination of glucose broth cultures of *Cl. paraputrificum* with *Cl. capitovalis* serum which may be considered as equivalent to normal serum, resulted from the action of acid on some substance of the broth infusion, rather than on the serum or bacteria present. Similar reactions were obtained with *Cl. capitovalis* and *Cl. difficilis* which further confirmed the non-specificity of this type of agglutination. These results may explain the discrepancies obtained by Zeissler (1919) and Heller (1920) who condemned the agglutination test for differentiating the sporulating anaerobes.

SUMMARY

Non-specific serum agglutination of glucose broth cultures of *Clostridium paraputrificum* resulted from the action of the acid, produced by the fermentation of the glucose, flocculating in the infusion broth some substance which in turn brought down the bacteria. Acid agglutination of broth cultures of *Cl. paraputrificum* was very close to pH 5.1 in the presence of normal serum in 1:10 dilution, and occurred more or less completely below pH 4.8. The acid agglutination of watery suspensions of *Cl. paraputrificum*, according to the technique of Michaelis, covered a zone of pH 3.01 to 2.20 which further indicated that the non-specific reactions with *Cl. paraputrificum* resulted from the action of the acid on the broth rather than on the bacteria. The non-specific reaction with glucose broth cultures of *Cl. paraputrificum* and other sporulating anaerobes can be avoided by using neutral broth or physiological saline suspensions. It is possible that the non-specific agglutination of acid broth cultures of certain bacteria may explain the failure of some investigators to secure specific reactions in serologic agglutination tests.

REFERENCES

BENIASCH, M. 1912 Ztschr. f. Immunitätsforsch., **12**, 268.
FILDES, P. 1925 Brit. Jour. Exper. Path., **6**, 91.
FISCHER, B. 1928 Centbl. f. Bakt., Orig. I, **106**, 417.
GIESZCZYKIEWICZ, M. 1916 Ztschr. f. Immunitätsforsch., **24**, 482.
HALL, I. C., AND STARK, N. 1923 Jour. Inf. Dis., **33**, 240.
HALL, I. C., AND SCOTT, A. L. 1931 Jour. Bact., **22**, 375.
HALL, I. C., AND SNYDER, M. L 1934 Jour. Bact., **28**, 181.
HELLER, H. H. 1920 Jour. Inf. Dis., **27**, 385.
DE KRUIF, P. 1921 Jour. Gen. Physiol., **4**, 639, 655.
KRUMWIEDE, C., AND PRATT, J. 1912 Ztschr. f. Immunitätsforsch., **16**, 517.
MCILVAINE, T. G. 1921 Jour. Biol. Chem., **49**, 183.
MICHAELIS, L. 1911 Deut. med. Woch., **37**, 969.
MICHAELIS, L. 1917 Deut. med. Woch., **48**, 1506.
ROBERTSON, M. 1916 Jour. Path. and Bact., **20**, 327.
SNYDER, M. L., AND HALL, I. C. 1935 Centbl. f. Bakt., Orig., I, **135**, 290.
ZEISSLER, J. 1919 Berl. kl. Woch., **56**, 107.

CORRELATIVE STUDIES OF MICROSCOPIC AND PLATE METHODS FOR EVALUATING THE BACTERIAL POPULATION OF THE SEA[1]

MARGARET HOTCHKISS AND SELMAN A. WAKSMAN

Woods Hole Oceanographic Institution; Department of Bacteriology, New York Medical College and Flower Hospital, and Department of Soil Microbiology, New Jersey Agricultural Experiment Station

Received for publication May 18, 1936

An estimation of the abundance of marine bacteria offers technical difficulties because many of the organisms cannot be cultivated readily by the usual methods. Discouraged by the small numbers of bacteria found by the common plating methods, direct microscopic counts have been attempted by several investigators. A direct and accurate estimation of the bacterial population has not been possible even by these methods, the various concentration procedures seeming to increase the sources of error and the difficulties of technique. Recently Henrici (1933) has introduced a method for studying water bacteria by suspending slides in aquaria and then observing microscopically the forms which became attached; this method was later extended to the study of bacteria in natural bodies of water (1935). ZoBell has applied the method to the study of marine bacteria (1935). The method was not utilized for a quantitative study of the organisms and these workers questioned the feasibility of such a procedure.

Our original plan, to investigate the marine bacteria in the vicinity of Woods Hole by this direct method, was frustrated by the turbulence of the waters with their heavy suspensions of organic and inorganic particles. On slides suspended in sea water under laboratory conditions for varying time intervals a variation in the number of attaching bacterial cells was observed; a tech-

[1] Contribution No. 106 of the Woods Hole Oceanographic Institution; contribution from New York Medical College and Flower Hospital, and Journal Series Paper of the New Jersey Agricutural Experiment Station.

nique was evolved for making a quantitative count by this method, and it has been possible to establish a relation between such a determination and the numbers obtained by the plate method. In spite of the many errors in sampling and dilution, the plate method seems still the simplest method that is now available, especially since results of different workers in different localities can be reproduced.

The experiments of Waksman and Carey (1935, a and b) on the rate of bacterial multiplication in resting water have been repeated, using, for comparative purposes, the plate method, the direct microscopic method, and the new method which can be called the "attachment count." The technique used in the plate count has been described in previous publications (Reuszer, 1933; Waksman, 1934). For the direct microscopic count a modification of the method of Winogradsky (Waksman, 1932) was used.

In the "attachment count" method, glass microscope slides were boiled in soap and water, rinsed and immersed in chromic acid cleaning solution, again rinsed thoroughly in distilled water and placed in ethyl alcohol. Before use, the slides were passed through a flame and the alcohol allowed to burn off. The cleaned slides were then suspended for twenty-four hours in the solution to be tested. On removal, the slides were allowed to dry in a level position, fixed with methyl alcohol or heat, and stained with carbol-erythrosin. (Staining may be accomplished after a few minutes over a steam bath or in cold solution after one hour.) The slides were washed gently but thoroughly, so that no small needle-like crystals would complicate the microscopic picture. The excess moisture may be drained off, but the film is not to be blotted. The area to be counted was standardized with a micrometer eye-piece. For these experiments, a microscope field with an estimated area of 0.0066 sq. mm. was used. For counting, an oil immersion lens and a No. 10 ocular were employed. If the cells tended to have an even distribution on the slide, ten fields were counted; when the distribution was uneven a greater number of fields was examined. The counts were averaged to represent the total number of cells in 0.066 sq. mm. of surface.

The values obtained by the three methods were taken from

experiments designed to show, (a) the influence of temperature upon the attachment of bacteria to slides, (b) the influence of the addition of nutrient material upon the attachment of bacteria to slides, (c) the attachment of pure cultures of marine bacteria, and (d) the influence of filtration and light. A series of counts was also made on samples of sea water obtained weekly at two stations.

Statistical methods were used in an attempt to analyze the values obtained. In table 1, the data obtained by the plate count method have been compared with the values obtained by the attachment method. The subject, designated by (*s*), consisted of a series of eight groups representing numbers from 0 to

TABLE 1

Correlation between plate counts, in bacteria per cubic centimeter (s), compared with attachment counts, in cells per 0.066 sq. mm. (r)

GROUPS (*s*)	GROUPS (*r*)								
	0-75	250	500	1,000	2,000	5,000	10,000	30,000	Total
0–100	4	5		1	1				11
500	4	10	4	2					20
5,000	4	4	2		1				11
50,000	15	10	2	3	1	3			34
100,000	2	4	6	1	3				16
1,000,000	3	8	14	5	4	1	1		36
50,000,000	1	4		5	2	2	4		18
500,000,000						1	1	3	5
Total.....	33	45	28	17	12	7	6	3	151

500,000,000, and represented the bacteria per cubic centimeter estimated by the plate method. The relative, designated by (*r*), consisted of a series of eight groups representing numbers from 0 to 30,000, and indicated the bacteria per 0.066 sq. mm. estimated by the attachment method. As the figures for the plate counts were checked off entries were made in the corresponding square in the attachment count. Hence, reading left to right in the upper line where the plate count was between 0 and 100, it is seen that four times the attachment count was in the group 0 to 75, five times in the group 76 to 250, once in the group 499 to 1000, and once in the group 999 to 2000. Reading left to right on

the last line, where the plate count was 50,000,000 to 500,000,000, the attachment counts also appear in the columns indicating high figures. For a perfect direct correlation the line of squares

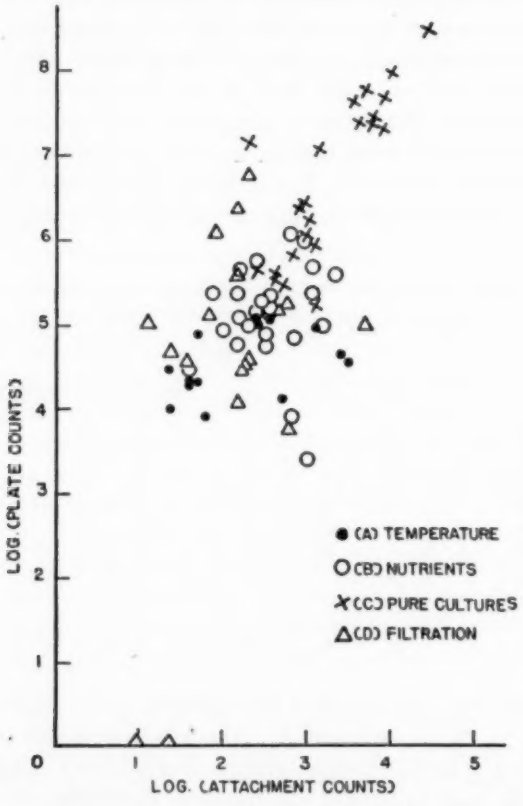


FIG. 1. SCATTER DIAGRAM. LOGARITHMS OF PLATE COUNTS PLOTTED AGAINST LOGARITHMS OF ATTACHMENT COUNTS

containing numbers should slope to the right at an angle approaching 45 degrees.

Similar tables (tables 2 and 3) were prepared to estimate any relation between the values obtained by the plate method and the direct microscopic method, and the counts obtained by the two

microscopic methods. All the data were used in making these tables. The tables suggest that some relationship may exist between the different values; this is more evident in the case of the attachment count than the direct microscopic count.

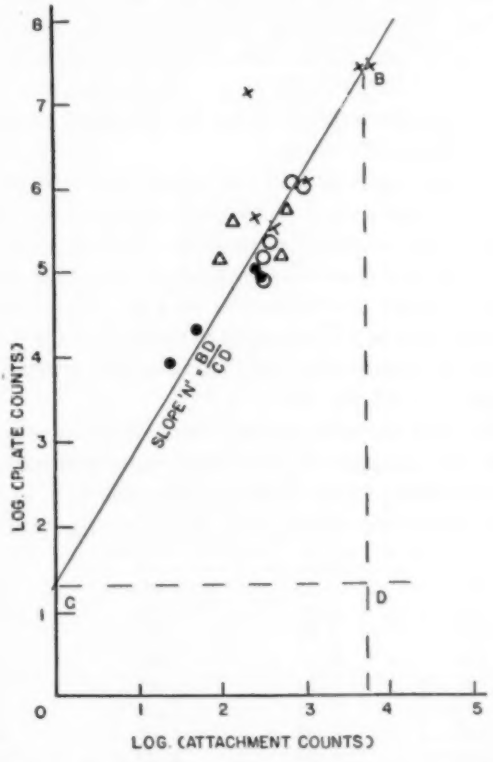


FIG. 2. LOGARITHMS OF PLATE COUNTS PLOTTED AGAINST LOGARITHMS OF ATTACHMENT COUNTS. VALUES FOR TWENTY-FOUR- AND FORTY-EIGHT-HOUR INCUBATION PERIODS

The most significant fact is the impossibility of grouping the numbers in an arithmetical series. In the scatter diagram (fig. 1), the logarithms of the plate counts (y axis) have been plotted against the logarithms of the attachment counts (x axis), and for this scatter diagram the results on resting water were used,

omitting the counts from the weekly sea water samples. A study of the graph suggests that there is a linear relation between the two logarithms of the counts.

If Y represents the plate count and X the attachment count, then by plotting log Y against log X, a mean straight line can be drawn which approximately represents the relation between these two variables. Hence the relation between the two types of count must be of the form $Y = AX^n$, where n is given by the slope of the straight line and A by its intercept on the log (plate count) axis (Feldman, 1935).

In figure 2 the logarithms of the values obtained in the twenty-four- and forty-eight-hour incubation periods have been plotted and such a mean straight line drawn. The slope of the line BC may be determined from the tangent of the angle BCD, and by direct measurement it is found to be 1.5. The intercept of the line BC has a value of 1.3, hence from figure 2, log A is 1.3.

Expressed in logarithms the fundamental equation becomes: $\log Y = \log A + 1.5(\log X)$.

The value to be assigned to "A" can also be calculated by using the values for X and Y obtained experimentally. Thirteen experimental values taken from experiments a, b, and c (incubation periods one to five days) gave the following results for A:

log Y	log X	log A	A
3.973	1.397	1.879	75.7
4.330	1.672	1.822	66.4
4.982	2.477	1.268	18.6
5.079	2.392	1.491	31.0
5.096	2.394	1.505	32.0
4.929	2.536	1.125	13.4
5.380	2.588	1.498	31.5
5.193	2.488	1.461	28.9
7.478	3.826	1.739	54.9
7.439	3.639	1.982	95.9
6.096	3.048	1.524	33.4
5.567	2.612	1.649	44.6
5.670	2.424	2.034	108.0
Mean		1.687	48.7

To test the equation, there remains a substitution of the calculated values of log A (1.687) and "n" (1.5) in the formula, and a

substitution of the observed value for log Y, using each experimental value found for Y. The value for log X can be calculated and a comparison with the value for X actually found can be

TABLE 2

Correlation between plate counts, in bacteria per cubic centimeter (s), compared with direct microscopic counts, in cells per 0.066 sq. mm. (r)

GROUPS (s)	GROUPS (r)								
	0–75	250	500	1,000	2,000	5,000	10,000	30,000	Total
0–100	7					1			8
500	13	1							14
5,000	10		1						11
50,000	15	10	2	4	1	1			33
500,000	13	18	9	3	2				45
5,000,000	10	2	2						14
50,000,000	1	1	1	1	3	1			8
500,000,000				1	2		3		6
Total.....	69	32	15	9	8	3	3		139

TABLE 3

Correlation between direct microscopic counts (s) compared with attachment counts (r), in cells per 0.066 sq. mm.

GROUPS (s)	GROUPS (r)									
	0–75	150	250	500	1,000	2,000	5,000	10,000	30,000	Total
0–75	22	11	7	10	8	2				60
150	4	2	3	3	4	1	1			18
250	3	1	4	5	2	2				17
500	2	1	1	6	1	4	1			16
1,000	2				1	3		2		8
2,000		1	1		1		3	3		9
5,000			1			1		1	1	4
10,000									2	2
30,000										
Total.....	33	16	17	24	17	13	5	6	3	134

made. The observed and calculated values are given in tables 4, 5, 6, and 7. Forty-nine of the 84 calculated values are in agreement with the observed values.

TABLE 4

Effect of temperature on sea water cultures. Attachment count, calculated and observed

INCUBATION	2 DAYS		5 DAYS		11 DAYS		15 DAYS	
	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed
1. Sea water 4°C.	33	25	188	248	195	354	112	174
2. 22°C. (dark)	58	47	80	3,184	157	1,359	80	52
3. 22°C. (light)	157	300	39	518	57	39	51	29
4. 30°C.	183	247	145	57	71	24	57	43

TABLE 5

Effect of added ingredients on sea water cultures. Attachment count, calculated and observed

INCUBATION	1 DAY		4 DAYS		9 DAYS		13 DAYS	
	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed
1. Sea water	145	344	264	286	155	1,614	128	275
2. Sea water + 1 mgm. glucose	219	308	304	150	130	681	459	169
3. Water + 3 mgm. glucose	291	383	168	201	301	1,120	533	256
4. Water + 1 mgm. glucose + 0.625 mgm. $NaNO_3$	820	942	479	1,201	203	362	108	354
5. Water + 3 mgm. glucose + 1.25 mgm. $NaNO_3$	857	690	447	2,180	236	280	118	149

TABLE 6

Growth of pure cultures of marine bacteria in sea water and in sea water plus peptone. Attachment count, calculated and observed

INCUBATION	1 DAY		3 DAYS		8 DAYS		13 DAYS	
	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed
1. Bacterium 16 (sea water)	867	1,118	1,450	872	347	553	652	1,228
2. Bacterium 22 (sea water)	386	410	555	682	449	486	231	1,273
3. Bacterium 23 (sea water)	455	266	1,520	896	4,210	1,380	1,150	1,022
4. Bacterium 16 (peptone)	4,400	208	7,250	6,290	13,000	5,766	9,780	3,275
5. Bacterium 22 (peptone)	7,020	6,702	38,600	26,000	17,300	11,508	17,300	9,200
6. Bacterium 23 (peptone)	6,830	4,362	19,800	26,000	14,000	2,261	13,900	9,300

The tables show that the mathematical agreement is best in the first three days of incubation, which represents the logarithmic stage of growth. During the next stage of growth the viable cells, represented by the plate counts, may be fewer than the cells which are visible under the microscope. Finally, in the longer incubation periods, an equilibrium is again established and shows itself in a mathematical agreement. A growth curve based on the attachment counts will be smoother than one based on petri plate counts, but the latter will be more sensitive to short time fluctuations.

TABLE 7

Effect of filtration and light on sea water cultures. Attachment count, calculated and observed

INCUBATION	2 DAYS		5 DAYS		10 DAYS		20 DAYS	
	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed
1. Sea water (dark)	197	74	71	181	43	157	149	104
2. Sea water (light)	209	531	134	219	198	219	90	222
3. Sand filtered (dark)	508	519	78	191	184	5,440	167	13
4. Sand filtered (light)	426	139	90	38	114	702	97	25
5. Seitz filtered (dark)	0	26	0	0	2,466	208	1,480	157
6. Seitz filtered (light)	0	10	7	0	871	99	151	270

In cultures containing many millions of cells per cubic centimeter the attachment count may become less accurate due to the difficulty in counting the microscopic field. Small cells are difficult to count and may cause discrepancies. Where concentrations of bacteria are low, and no variable which changes cell viability with rapidity has been introduced, the values reached by the two methods are comparable. (Such a variable might be a disinfectant which killed but did not disintegrate the cells.)

Conversely, if with adequate determinations, no mathematical agreement can be reached by the two methods, an effort should be made to detect some unrecognized factor which is influencing the growth of the cells.

In clear waters, where preparations may be made by suspending

slides directly, it should be possible to obtain comparable values; but when samples must be taken and kept in the laboratory for the twenty-four-hour period a second plate count is necessary so that an interpolation may be made to determine the number of viable cells originally present.

SUMMARY

Bacterial numbers may be estimated by the immersion of slides in water, with a slight modification of Henrici's original technique. In contrast to other microscopic methods, this method, designated as the attachment count, can be utilized in the case of waters containing small numbers of bacteria.

Not only were high values for the plate counts associated with high values for the attachment counts; but constants could be determined from experimental data which permitted the calculation of the numbers to be expected from the attachment count, if the plate counts were known.

The correlation between the values obtained by the two methods was noted in a study of a series of growth curves obtained by using, (a) sea water samples kept at different temperatures, (b) sea water samples to which a source of carbon or a source of carbon and nitrogen in combination was added, (c) pure cultures of marine bacteria growing in sterile sea water or in sea water enriched with peptone, (d) filtered sea water.

REFERENCES

FELDMAN, W. M. 1935 Biomathematics. London.
HENRICI, A. T. 1933 Jour. Bact., **25**, 277–286.
HENRICI, A. T. 1935 Jour. Bact., **30**, 61–92.
REUSZER, H. W. 1933 Biol. Bull., **45**, 480–497.
WAKSMAN, S. A. 1932 Principles of Soil Microbiology. 2d ed. Williams & Wilkins, Baltimore, Md.
WAKSMAN, S. A. 1934 Ecological Monographs, **4**, 523–529.
WAKSMAN, S. A., AND CAREY, C. L. 1935a Jour. Bact., **29**, 531–543.
WAKSMAN, S. A., AND CAREY, C. L. 1935b Jour. Bact., **29**, 545–561.
ZOBELL, C. E., AND ALLEN, E. C. 1935 Jour. Bact., **29**, 239–251.

CHANGES IN THE ELECTROKINETIC POTENTIAL OF BACTERIA AT VARIOUS PHASES OF THE CULTURE CYCLE

LAURENCE S. MOYER[1]

Department of Public Health, Yale School of Medicine, New Haven, Connecticut

Received for publication, May 22, 1936

INTRODUCTION

The investigations of Schultz and Ritz (1910), Reichenbach (1911), Sherman and Albus (1923, 1924), Heiberg (1932), and Sherman and Cameron (1934) have demonstrated that bacteria from freshly made cultures are killed by various agents and temperatures which are innocuous to older cells. Bayne-Jones and Rhees (1929), and Schmidt and Bayne-Jones (1933) have shown an increased rate of heat production per cell during this stage of "physiological youth." Martin (1932), Walker, Winslow, Huntington and Mooney (1934), and Mooney and Winslow (1935) have reported an increased O_2 consumption and CO_2 and NH_3 yield at the same period. In the light of these results, it is obviously a problem of considerable importance to investigate the changes which the bacterial surface may undergo during this phase of the culture cycle. Any alterations in surface properties might be reflected by changes in the electrokinetic potential unless some peculiar set of circumstances were to keep the surface charge density constant.

Although the literature of bacterial electrophoresis is voluminous (cf. Abramson, 1934a), no systematic investigation of the influence of the age of the culture on the electrokinetic potential of its cells has been carried out under standard conditions. In 1924, Shibley found that young cells of *Pneumococcus* Type I and

[1] Sterling Fellow, Yale University, 1935-36; present address, Department of Botany, University of Minnesota.

Salmonella Schottmuelleri move more slowly in an electric field than older cells. Since then, Buggs and Green (1935) working with *Escherichia coli* and *Staphylococcus aureus* report no change in the electrophoretic mobility of cells from 6 hours to 10 days after seeding. Pedlow and Lisse (1936) likewise claim that cells of *Escherichia coli* do not vary in mobility even down to the third hour after seeding.

In this communication, evidence will be presented which has been obtained from organisms grown and measured under carefully controlled conditions and which indicates that the surfaces of certain bacteria are greatly modified during the youth of the culture. Furthermore, the data have been treated by the modern theory of electrolytes, giving quantitative evidence of the soundness of the assumptions underlying the theory of Gouy and, in addition, throwing new light upon the chemical constitution of bacterial surfaces. It is shown that these changes in the surface which occur during the population cycle are intimately linked with the growth process and, in particular, with the periods of lag and "physiological youth."

METHODS

Bacterial strains. Most of the investigations were done with the same strain of *Escherichia coli* (*communis*) which has been used in previous studies from this laboratory. Rough variants were obtained as described later. In addition, some observations were made on the strain of *Salmonella gallinarum* used by Mooney and Winslow (1935). Stock cultures, unless otherwise noted, were grown on meat-extract agar slants. Cultures for growth studies were grown in peptone water (1 per cent Difco bacto-peptone), usually in Drechsel bottles at 37.0°C. in a water-bath, and aerated with CO_2- and NH_3-free air after the method of Walker (1932). This was done to conform with previous reports from this laboratory. In most cases the technique followed that of Winslow, Walker and Sutermeister (1932) and Walker (1936). Details of seeding and any variations in methods are described in connection with the experiments.

Electrophoretic measurements. All measurements of electro-

phoresis were made in the Abramson microelectrophoresis instrument employing the technique described in detail by Moyer (1936). Field strength was kept constant throughout any series of measurements on a given suspension and mobilities were calculated by the use of Ohm's law. The depth of the chamber was measured each time a new suspension was introduced. All measurements were corrected to 25°C. (Moyer, 1936). Since the influence of crowding on the mobility, if any, is unknown, all measurements were made on dilute suspensions. Unless mentioned otherwise, twenty organisms (ten at each level) were measured for any one mobility value. Throughout this paper, it is to be understood that all bacteria were negatively charged under the conditions employed.

Buffers. For most of the work two buffer concentrations were used. These were, (1) acetic acid-sodium acetate solution at pH 5.5 and an ionic strength[2] of 1/50 and (2) Sørensen's phosphate buffer at pH 6.9 and $\mu = 1/75$ (or M/150 phosphate). The unqualified mention of phosphate or acetate buffer means these particular dilutions and pH values. The Na_2HPO_4 and KH_2PO_4 were of Merck reagent grade. Salts of lower quality containing traces of divalent ions caused great divergence. All pH values were checked with the quinhydrone electrode. A simple vacuum tube oscillator[3] and Wheatstone bridge were used in the determination of specific resistances. Further technical details are to be found in the experimental section.

EXPERIMENTAL RESULTS WITH ESCHERICHIA COLI

Separation of rough and smooth strains

Joffe and Mudd (1935) have reported that certain smooth intestinal bacteria have no electric mobility at any pH value,

[2] Ionic strength is defined as

$$\mu = \tfrac{1}{2} \Sigma c_i z_i^2,$$

where c_i is the concentration of the i-th ion and z its valence. In practice, the concentrations (in mols per liter) and squared valences of each ion are added and the sum divided by 2. In the case of uni-univalent strong electrolytes, μ is equal to the molarity.

[3] For the loan of certain instruments and assistance in the construction of the oscillator, I am greatly indebted to Dr. L. W. McKeehan and Dr. C. T. Lane.

whereas the corresponding rough variants possess a high mobility which is dependent on pH. This fact makes it imperative to make sure of the homogeneity of one's cultures before beginning electrophoretic investigations. As mentioned by Winslow, Walker and Sutermeister (1932), the strain of *E. coli* used was of mixed R and S forms. Although Reed and Gardiner (1934) have described

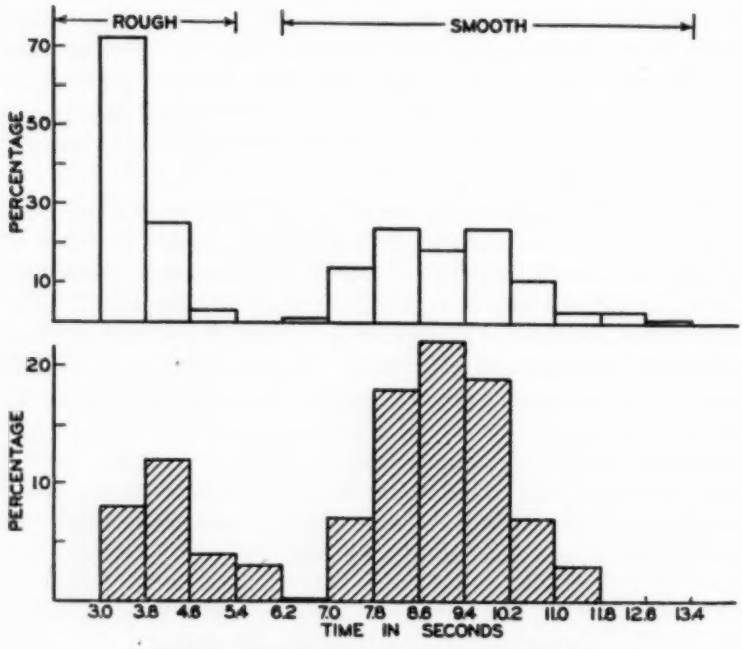


FIG. 1. HISTOGRAM OF MOBILITIES OF ESCHERICHIA COLI

Lower, mixed culture of rough and smooth types. Upper, separate curves for pure rough and pure smooth forms after plating. Time values represent time required to travel a certain distance under constant field strength.

a macroscopic method for isolating R and S forms from mixed culture by electrophoresis, there appear to be no quantitative data on the electrophoretic behavior of such mixed cultures. Consequently a 16-hour slant was washed off into phosphate buffer and centrifuged and resuspended three times. One hundred cells were timed, taking care to make the measurements as objective as possible. The results are shown in figure 1 (lower).

The bimodality of the frequency histogram will be noted. Since mobility varies inversely with the time values, the time values required for a particle to travel a certain distance are plotted; otherwise the data would be unwarrantably weighted at the extremes and appear skew. It has been our practice to transform the mean time values into terms of mobility rather than to transform each value separately. All measurements in figure 1 are at constant field strength.

The culture was then streaked on Endo's agar plates and colonies of typical rough and smooth appearance selected. Both R and S forms gave the usual biochemical reactions typical of *E. coli*. These variants were then replated separately, fished, and grown for 16 hours on extract slants. Upon measurement of 50 cells from each culture, the type picked as rough yielded time values extending between 3.0 and 5.4 seconds whereas the smooth form ranged from 6.2 to 13.4 seconds. A comparison with the lower graph (fig. 1) shows that R and S forms preserve their individuality of mobility even in mixed culture. The histogram of the mixed form bears a marked resemblance to other electrophoretic histograms depicted in the literature and suggests the need for clearly separating R and S variants.

Reproducibility of results

Traces of the culture medium adsorbed on the surfaces of bacteria may greatly influence their behavior. As Northrop and de Kruif (1922) have pointed out, washing by centrifugation is necessary for quantitative, reproducible measurements. Obviously some culture media will be more readily removed than others (especially those containing unsplit proteins). To develop a standard washing technique for the present conditions, samples from 24-hour aerated peptone cultures of *E. coli* S were centrifuged for 15 minutes at 3500 r.p.m. in an angle centrifuge and resuspended in phosphate (and in the second experiment, in acetate) buffer. This was designated as 0 washing. A portion having been withdrawn for measurement, the rest was centrifuged and resuspended three times, taking out a sample each time. As shown in table 1, mobility values did not change much from 0 to 3 washings with either buffer. When the 40 measurements on each

sample were analyzed statistically, they showed a marked decrease in the coefficient of variation[4] on the first washing but changed little with further rewashing. In all further experiments the samples were each washed twice (i.e., resuspended in buffer 3 times). As stated before (Moyer, 1936), suspension in distilled

TABLE 1

The effect of successive washings with buffers upon the uniformity of electrophoretic behavior of E. coli S

Figures represent averages of 40 measurements

NUMBER OF WASHINGS	PHOSPHATE, pH 6.9		ACETATE, pH 5.5	
	Mobility	Coefficient of variation*	Mobility	Coefficient of variation*
	μ/sec./v./cm.	per cent	μ/sec./v./cm.	per cent
0	0.76	18.4	0.70	13.6
1	0.81	10.7	0.68	6.1
2	0.79	10.8	0.69	6.7
3			0.69	7.8

* Calculated from mean time values.

TABLE 2

Effect of growing the same strain of E. coli under different conditions for a year

The smooth form was recovered in each case by plating. Figures represent averages of 40 measurements in phosphate buffer, pH 6.9.

STOCK	MEDIUM	MOBILITY	COEFFICIENT OF VARIATION*
		μ/sec./v./cm.	per cent
A	Extract + infusion	0.92	10.1
B	Extract only	0.89	10.5
C	Extract only	0.91	13.2

* Calculated from mean time values.

water yielded about 16 per cent for the coefficient of variation, even after 3 washings. Once suspended in either of the two buffers, however, no significant changes could be noted for at least 2 to 3 hours. In two experiments designed to investigate this point, the greatest deviation in mobility was but 6 per cent.

[4] Defined as the standard deviation of a single observation divided by the mean and expressed as per cent.

The literature of bacterial electrophoresis frequently gives one the impression that bacteria vary so rapidly and so greatly that no significance can be attached to comparative measurements made on the same strain at different times. An opportunity to test this idea presented itself. One year before these experiments began, the strain of *E. coli* had been divided up into several stock cultures, as follows: A, grown on extract agar but once a week "rejuvenated" by a transfer on meat-infusion agar, B and C, grown on extract agar only. Each of these stock cultures was now plated and the smoothest-appearing colony on each plate was fished. Table 2 shows the mobilities of organisms from each of these cultures grown for 16 hours on extract slants. It is clear that the smooth form maintained its identity and could be recovered by plating even when rough forms were present. In the rest of this report, the *E. coli* S was from stock B, in which rough forms had never been found.

Changes in mobility with age of the culture

Unaerated cultures. Experiments were first performed with unaerated peptone cultures of *E. coli* S grown in test tubes. Preliminary measurements showed that variations in the area of the surface of the culture exposed to air could produce marked changes in growth. Precautions were therefore taken to use pyrex test tubes of uniform bore. The seed culture was grown for 16 hours on an extract slant and then washed off into 12.5 cc. of 1 per cent peptone. From this suspension, 1 cc. was used to inoculate each culture tube containing 10 cc. of peptone solution. The resultant 11 cc. contained approximately 100 to 150 million bacteria per cubic centimeter (checked by plating). The tubes were plugged with cotton and incubated in an air bath at 37°C. They were not shaken. At suitable intervals, the contents of a tube were centrifuged, washed with acetate buffer and the mobility determined. It was found that better agreement was secured if the age of the culture was plotted as the time at which the peptone was poured off and the first change of buffer added in centrifuging, i.e., 15 minutes after leaving the incubator. Growth probably continues for nearly that length of time in the cen-

trifuge. Points plotted as "0" hours in figure 2 are from 16-hour extract slant cultures suspended directly in the buffer, washed and then measured; those plotted as 0.25 hours were obtained by washing and measuring the rest of the suspension used as seed.

As will be noted from the different kinds of circles, cultures from the several days' runs showed no significant differences. In each case, the electric mobility starts dropping immediately and continues to drop for an hour; the same lowered mobility is held until 4 hours of age. From this point the curve rises to the initial, higher value at 8 hours, which value it retains from then on.

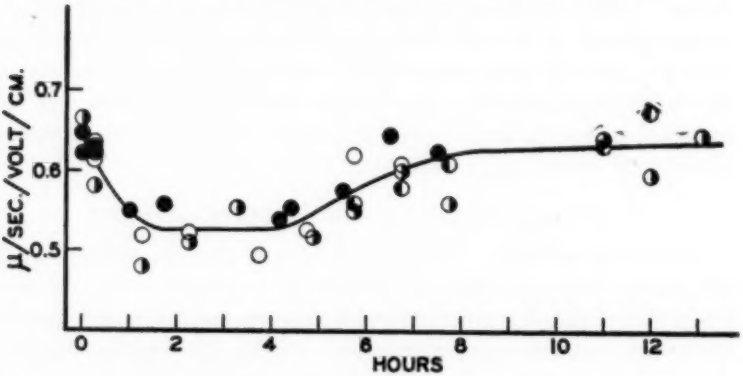


FIG. 2. ELECTROPHORETIC MOBILITIES OF BACTERIA FROM UNAERATED E. COLI CULTURES PLOTTED AGAINST AGE OF CULTURE

Different circles represent experiments done on different days. M/50 acetate buffer, pH 5.5.

Although the drop is only about 18 per cent, it is quite definite and no exceptions were found. The scatter of points is fairly great, probably because a separate culture was used for each point.

Aerated cultures. The smooth form of *E. coli* was grown in Drechsel bottles and aerated as described in the section on methods. The seeding technique of Winslow, Walker and Sutermeister (1932) was used but the seed suspension in distilled water was unfiltered. The concentration of the medium and suspension was so arranged that the addition of 10 cc. of the distilled water suspension to 90 cc. of the peptone solution produced a

suspension of from 15 to 20 million bacteria per cubic centimeter in 1 per cent peptone. Figure 3 (lower) shows the electrophoretic behavior of these cultures in the acetate and phosphate

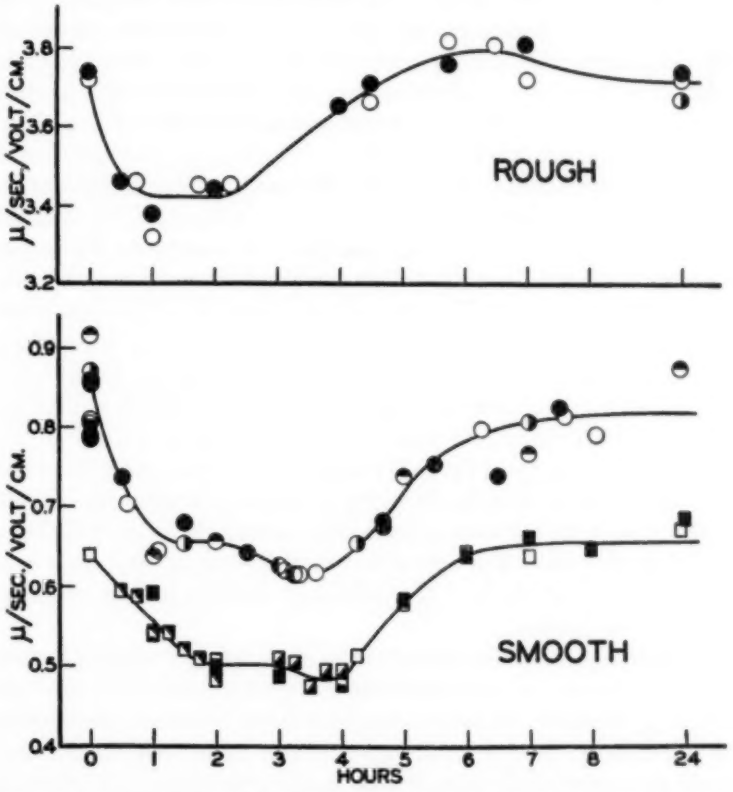


FIG. 3. ELECTROPHORETIC MOBILITIES OF BACTERIA FROM AERATED E. COLI CULTURES PLOTTED AGAINST AGE OF CULTURE

Upper curve: rough form in M/50 acetate buffer, pH 5.5. Lower curves: smooth form measured in M/150 phosphate (circles) and M/50 acetate (squares) buffers at pH 6.9 and 5.5 respectively. Different markings represent experiments done on different days.

buffers. The values for the different days' runs are indicated by the different circles and squares. The zero-hour values are not so constant as the rest; they were from organisms suspended

directly in buffer from a slant and were probably less completely freed from metabolites. The agreement between points is much better with the aerated cultures and the drop in mobility is greater (*ca.* 27 per cent). The phosphate curve was done first and a few of the points are low, probably due to errors in focussing. The secondary dip in both curves at 3 to 4 hours is believed to be real as it occurs so consistently. As anticipated, the acetate curve is always lower than the phosphate, because of the higher ionic strength of the menstruum.

It was found impractical to use the same technique for seeding cultures of the rough form because of the difficulty of producing homogeneously dispersed suspensions. If, however, the organisms were grown in aerated peptone for 24 hours, they became uniformly dispersed. Therefore 10 cc. from one of these cultures were added to 100 cc. of 1 per cent peptone, giving a resultant concentration of from 90 to 110 million bacteria per cubic centimeter. This was roughly 10 times the concentration used for the smooth form. Samples were suspended and measured in the acetate buffer to yield figure 3 (upper). It will be noted that this variant shows a definite decrease in mobility during the growth cycle (about 12 per cent) and a subsequent rise with possibly a slight maximum. The agreement between the several days runs is particularly striking. The absolute mobility values are some 500 per cent higher.

Although these experiments were completely consistent, they were all based on comparisons of several samples. It was decided, therefore, to take advantage of several other phenomena in order to make a crucial experiment. It is well known that great changes in size occur during the growth cycle. Huntington (1936) has recently presented data to show that *E. coli* reaches its maximum size at about 2 hours. As seen in the electrophoresis cell, bacteria from young cultures are markedly longer than those from old ones. Now it has been shown repeatedly (Abramson, 1934a) that if the salt concentration of the suspending medium is high enough, large particles in an electric field move independently of size and shape.

In comparing the electrophoretic behavior of mammalian

erythrocytes, Abramson (1929) found that red cells having closely similar mobilities could be successfully distinguished from each other by investigating a mixed suspension. In this way errors in focusing, concentration of buffer, and changes in temperature are made identical for the two types of cells. Young cells (1.5 hours after seeding) and old cells (16-hour, from extract agar, and 24-hour cells grown in 1 per cent aerated peptone) were each washed three times by centrifugation in the phosphate buffer. After washing, the 24-hour cells were mixed with the 1.5-hour cells (experiment 1). In addition, 1.5-hour cells were mixed with washed cells from the 16-hour culture used as seed (experiment 2).

It was found that the long, young cells moved more slowly than the short, old ones; in fact, the short cells overtook the long ones

TABLE 3

Variation in mobility with age of culture

Experiment in mixed culture. Explanation in text

EXPERIMENT	AGE	SIZE	TIME	MOBILITY
	hours		*seconds*	*μ/sec./v./cm.*
1	24	Short	4.73 ±0.11	0.88
1	1.5	Long	5.90 ±0.11	0.71
2	16	Short	4.98 ±0.11	0.86
2	1.5	Long	6.02 ±0.18	0.69

and passed them. No orientation of individual cells was seen at any time, in disagreement with Choucroun (1934). In each experiment, 25 long cells and 25 short cells were timed; the mean times together with their standard errors are shown in table 3. It will be noticed that the mobilities calculated from the mean time values show a difference of about 23 per cent between long and short cells in each experiment, but the deviation of long from long, and short from short, is but 3 per cent. In experiment 1, the difference between the two time values was found to be over seven times the standard error of the difference. In experiment 2 it was five times. Calculation of t and use of Fisher's t table (Fisher, 1930) gave the same result. If some of the young cells were still small enough to have been mistaken for older cells, it

would have tended to make the difference between the two sets of data less, not more. Furthermore, electrokinetic theory (Abramson, 1934a) demands that particles of identical surface constitution in a medium where κr is $\gg 1$ should move independently of size and shape.[5] If $\kappa r < 6$, large cells should, by theory (if size alone is the factor involved), move *faster* than small cells. In many experiments in which unmixed cultures were investigated in this buffer, it was found that the cells of 1.5 to 4 hours of age retain this same decreased mobility. During this time, although marked changes in size take place due to division, no influence of size or shape on mobility could be noted, indicating that either κr is large or else constant (Moyer and Abramson, 1936).

The cause of the change in the mobility with age

This regular alteration in the mobility of both *E. coli* R and S can only be interpreted as a change in the properties of the surface for ionic strength, ionic species, and pH were held constant. Several possibilities suggest themselves as causes of the lowered mobility shown by these bacteria between the ages of 1.5 to 4 hours. Among these are (1) irreversible changes in surface caused by the buffer, (2) an adsorption of dissolved metabolites, (3) the adsorption of gaseous metabolites, (4) the presence of flagella, or (5) an actual chemical or physical change in the surface. Some light can be thrown on these hypotheses.

Hypothesis 1: Changes due to the buffer. It might be argued that since young cells are more readily injured by strong salt solutions (Sherman and Albus, 1923, 1924) they are changed by the buffer so that the surface alters, either by leaching or by a denaturation of some sort. To test this we measured mobilities directly in fresh 1 per cent peptone. Fresh peptone was used to eliminate differences in viscosity and electrolyte concentration.

[5] Here r is the radius and

$$\kappa = \sqrt{\frac{4\pi \mathbf{N}\mathbf{e}^2}{1000\, D\mathbf{k}T}} \sqrt{\Sigma c_i z_i^2},$$

where $\mathbf{N}$ = Avogadro's number, $\mathbf{e}$ = the electronic charge, D = the dielectric constant, $\mathbf{k}$ = Boltzmann's constant, and T = the absolute temperature.

For routine work, it is important to emphasize that buffers are preferable because of their known ionic strength. Two experiments were performed in which aerated *E. coli* S cultures were centrifuged at three ages: 16, 4.25 and 6.25 hours. After decanting, the organisms were resuspended in fresh peptone and measured as quickly as possible to avoid any decrease in mobility caused by growth in the fresh medium. The electrophoresis cell was, as usual, first rinsed several times with the medium. No orientation or dependence of mobility on size or shape was noted. As shown in table 4, the young cells in each experiment were slower than either of the older samples. The two experiments should not, strictly, be compared because of possible changes due to growth in the new medium. Any growth would,

TABLE 4

Changes in mobility between old and young cells of E. coli S measured in fresh 1 per cent peptone

EXPERIMENT 1			EXPERIMENT 2		
Age	Mobility	Decrease	Age	Mobility	Decrease
hours	*μ/sec./v./cm.*	*per cent*	*hours*	*μ/sec./v./cm.*	*per cent*
16	0.79	27	16	0.70	27
4.25	0.58	17	4.25	0.52	29
6.25	0.70		6.25	0.73	

however, have *lessened* the differences. This shows that the decrease in mobility does not depend upon the buffer. The success of this experiment was due to the absence of easily adsorbable proteins from the peptone.

In addition, 1 cc. from a 4-hour aerated culture was suspended in 100 cc. of each of the two buffers. These were allowed to stand for one hour before plating while another sample was plated immediately. Based on this control, it was found that 114 per cent of the bacteria exposed to acetate and 34 per cent of those in phosphate were living at the end of the hour. Since there was no appreciable difference between the shapes of the acetate and phosphate curves (fig. 3), it is probable that injury caused by buffer does not produce marked changes in surface properties.

Hypothesis 2: Adsorption of metabolites. The bacteria might produce surface-active substances which are adsorbed on their surfaces during the 1.5–4 hour period and then eliminated after that time, or *vice versa*.

Adsorption (as the term is used here) is considered as an equilibrium phenomenon (Hückel, 1928); that is, some adsorbate is always present in the solution even after the surface has adsorbed all it can hold. If Hypothesis 2 is correct, it would be reasonable to expect that old cells placed in "young" broth (e.g., broth from a 3-hour culture) or young cells placed in "old" broth (e.g., 24 hours old) would adsorb these substances and converge toward the same mobility as that of young cells in "young" broth or old cells

TABLE 5

Experiment to show that young cells are relatively slower than old cells irrespective of the age of the medium

AGE OF CELLS	AGE OF BROTH	SPECIFIC CONDUCTANCE	MOBILITY	REMARKS
hours	*hours*	*Mho* $\times 10^4$	μ/sec./v./cm.	
24	24	14.7	0.67	Control
3	24	14.7	0.44	Old cells pass young
3	3	8.38	0.51	Control
24	3	8.38	0.91	Old cells pass young

in "old" broth respectively. Cultures of *E. coli* S were therefore centrifuged and the supernatant broths exchanged. A control experiment was performed for each (see table 5) at room temperature. As an index of the ionic strength of the medium, the specific conductance at 25°C. is given. It will be noted that in each case the young cells moved slower than old cells in broths of the same age. A few bacteria of each age were transferred accidentally with the broth but it could be clearly seen that young cells were overtaken by the old as in table 3. The mobilities in the two ages of broth cannot be directly compared because the specific conductance of the old broth is nearly double that of the young. Fresh 1 per cent peptone had a conductance of 7.11×10^{-4} mho at 25°C.

Collodion particles might adsorb these hypothetical substances more readily. These particles were suspended both in 1 per cent peptone and in the supernatant from a centrifuged 3-hour culture. The mobility values showed a difference of but 6 per cent which was probably not significant. If such substances were present, the bacteria could probably adsorb them for they adsorb gelatin readily (as will be seen later). Possibly there is a difference during the growth cycle in the ability of the surface to adsorb but, if so, the surface must have changed physically or chemically. The presence of the slight but consistent bend in the curve at 3 hours would seem to speak against metabolite adsorption.

Hypothesis 3: Adsorption of gases. Since the cells are respiring most rapidly during the early stages of growth (cf. Walker, Winslow, Huntington and Mooney, 1934), gaseous metabolites may be issuing from the surface at a steady rate so that the bacterium presents a surface which is essentially gaseous at that time. It is well known that gases are able to adsorb ions and possess electrophoretic mobilities quite similar to other "inert" surfaces (Abramson, 1934a). In this connection, Pilojan, Kriworutschko and Bach (1933) have shown that H_2 and O_2 adsorbed on carbon can markedly alter its electrophoretic behavior. To test this we washed young cells of the S form in buffer and then aerated the suspension thoroughly with CO_2- and NH_3-free air for an hour or more at 25°C. During this time, the outlet tube was connected to a CO_2 absorption train containing a solution of $Ba(OH)_2$ to collect any CO_2 formed while in the buffer, as an index of aerobic respiration. A partial pressure of $CO_2 = 0$ and a pH of 5.5 should have tended to release any CO_2, for if CO_2 was the sole cause of this phenomenon, it could not be firmly adsorbed, since the mobility rises again after 4 hours. As will be seen in table 6, both the control and exposed samples retained their same lowered mobility. Furthermore, not a trace of CO_2 could be detected in the $Ba(OH)_2$. In addition, the curves for CO_2 and NH_3 production of Walker, Winslow, Huntington and Mooney (1934) do not reach their maxima at 3.5 hours but at about 1.5 hours. Furthermore, both R and S variants retain their extremely divergent mobilities

although respiring rapidly. These experiments indicate that CO_2 or other gases are not held tightly enough to be involved in the lowered mobility.

Hypothesis 4: Flagella. As Joffe and Mudd (1935) emphasize, workers in this field have made little or no attempt to eliminate variations due to R and S forms and to the presence or absence of flagella. It is well known in immunology that the presence of the H antigen carried by the flagella markedly complicates serological behavior and, concomitantly, surface properties (Craigie, 1931, Kauffmann, 1934, Hornus, 1935). Consequently it might be assumed that the surface behavior was masked by the presence of the H antigen during certain periods of the life cycle. It has

TABLE 6

Effect of aerating suspensions of E. coli S held in buffers for 1 to 2 hours

AGE	BUFFER	pH	AERATION TIME	CO_2 FOUND	MOBILITY	CHANGE ON AERATION
hours			*hours*		*μ/sec./v./cm.*	*per cent*
3	Phosphate	6.9	0		0.62	Control
3	Phosphate	6.9	1	None	0.64	3.1
4	Acetate	5.5	0		0.46	Control
4	Acetate	5.5	2	None	0.48	4.3
4	Acetate	5.5	2	None	0.47	2.2

been shown by Braun and Schaeffer (1919) and later confirmed by both White (1926) and Craigie (1931), among others, that H antigen and flagella are completely inhibited in bacteria grown in culture media containing 0.1 per cent phenol. These authors emphasize that the O antigen is not injured in any way by the treatment, although growth is slightly inhibited. Use was made of this discovery to test the hypothesis. A culture of *E. coli* S was grown in peptone and on slants which contained phenol of the proper concentration. The resultant growth was slower than usual because of the toxic effects of the phenol but the electrophoresis curve, as shown in figure 4, dropped and rose again. The normal curve for organisms in the same buffer is also shown; both curves have been continued for some time longer than in

figure 3 but with no further changes in mobility. The phenol curve is slower in falling and rising but does not appear to be otherwise significantly different.

White (1926) and Craigie (1931) both agree that lack of motility is a good criterion for absence of flagella. Although special efforts were made to observe these organisms under normal conditions at all phases of growth, no motility could be detected in either the R or S forms. It therefore seems safe to assume that long cultivation under laboratory conditions has caused the strain to lose any flagella it may have possessed.

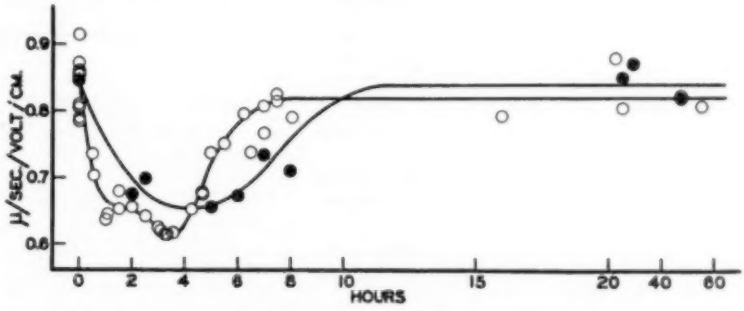


FIG. 4. ELECTROPHORETIC MOBILITIES OF BACTERIA FROM AERATED E. COLI CULTURES PLOTTED AGAINST AGE OF CULTURE

Open circles: untreated cultures. Closed circles: cultures grown in 0.1 per cent phenol. M/150 phosphate buffer, pH 6.9. Note change in scale on time axis.

Hypothesis 5: An actual change in the surface itself. This appears to be the simplest and most reasonable assumption. Furthermore, it is in agreement with the results of experiments on the fragility of the organism at this time. Such a change may be a qualitative one or simply a quantitative rearrangement of the components of the surface. The reaction of the organism to heat at different ages should prove a useful test of this hypothesis. Joffe and Mudd (1935) heated their organisms (24 hour) to 56°C. for 0.5 hour while still suspended in the growth medium before washing. They found no change in electrophoretic mobility on heating, a conclusion in agreement with the findings of Winslow, Falk and Caulfield (1923) (also using old cultures). What is the result of heating young cultures?

Samples of various ages from our cultures of *E. coli* S were heated in a water-bath at 56°C. for 0.75 hour and then centrifuged. They were first washed three times with buffer and then timed. Table 7 shows that mobilities of the 24-hour cells were scarcely changed by heat (cf. also Buggs and Green, 1935) whereas 3-hour organisms suspended in phosphate buffer showed a marked scattering of individual mobilities over a range so wide that no definite value could be assigned. Those in acetate buffer evidenced only slight changes, although their mobilities were also more variable.

TABLE 7

Effect of heating cultures of E. coli S at different ages while in peptone to 56°C. for ¾ hour

EXPERIMENT	AGE	BUFFER	MOBILITY	DEVIATION FROM CURVES IN FIGURE 3
	hours		*μ/sec./v./cm.*	*per cent*
1	24	Phosphate	0.82	2.4
1	3	Phosphate	0.0–3.6	Great
2	24	Phosphate	0.75	8.0
2	3	Phosphate	0.0–3.4	Great
3	24	Acetate	0.60	7.7
3	3	Acetate	0.40	20
4	24	Acetate	0.59	1.7
4	3	Acetate	0.45	10

These results suggest a definite alteration of the surface during the early stages of the population cycle and indicate that the method of Joffe and Mudd may not be suitable for the routine investigation of the surfaces of young bacteria.

The charge density of bacterial surfaces

The electrophoretic mobility, v, of bacteria depends upon the electrokinetic potential, ζ, at their surfaces. It is generally agreed that the two quantities are related by the well-known Helmholtz equation,

$$\zeta = \frac{4\pi\eta}{D} v, \qquad (1)$$

where η is the viscosity and D the dielectric constant of the medium, all units being c.g.s. and e.s.u.

By the use of the theories of the diffuse double layer,[6] it may be shown that ζ is dependent on two factors, σ, the density of net charge at the surface[7] and λ, the statistical thickness of the ionic atmosphere surrounding the particle, through the expression,

$$\zeta = \frac{4\pi}{D}\sigma\lambda. \tag{2}$$

To evaluate σ, it is necessary to determine λ, the thickness of the double layer. When $z\zeta$ is small,[8]

$$\lambda \cong \frac{1}{\kappa} = \frac{4.32 \times 10^{-8} \text{ cm.}}{\sqrt{\Sigma c_i z_i^2}}, \tag{3}$$

at 25°C., where $\Sigma\, c_i z_i^2$ is equal to twice the ionic strength. In other words, the thickness of the double layer is affected solely by the ionic strength (at constant T).

Therefore ζ is not nearly so fundamental a characteristic of a surface as is σ, for it is bound up with the thickness of the double layer, which may vary independently of σ and which in no wise depends upon the nature of the surface. σ, on the other hand,

[6] The concept of Helmholtz, visioning a rigid electrical double layer at the surface of a particle suspended in a liquid has been replaced, through the work of Gouy, and Debye and Hückel, by the idea of a diffuse double layer. Instead of two layers of opposite sign at molecular distances, this theory demands that the outer layer, or ionic cloud, which is free to move, extends for an infinite distance out into the solution and possesses, over a time average, a net charge opposite in sign to the ions held by the particle. The electrical center of gravity of this cloud is generally referred to as the thickness of the double layer. The ions on the particle arise from either (1) preferential adsorption from the solution or (2) dissociation of adsorbed molecules or those of the surface itself. (Abramson, 1934a, Müller,1934a).

[7] It has been customary for writers to speak loosely of ζ as the "charge". In reality, this is not the case. Clear distinction should be drawn between σ, the net charge density per cm^2, ζ, the electrokinetic *potential*, and the net charge per cell. In this connection, see the discussion by Abramson (1935) and Abramson and Moyer (1936).

[8] This assumes that the Debye-Hückel approximation may be made, i.e., that the hyperbolic sine may be replaced by its argument in the solution of the equation

$$\nabla \cdot \nabla\psi = \frac{\kappa^2 kT}{e} \sinh \frac{e\psi}{kT}.$$

bears a relationship to the adsorption conditions prevailing at the surface of the cell (Müller, 1934b, Moyer and Bull, 1935).

Equation 2 serves as an illustration, but for larger values of ζ it is more accurate to employ the integrated equation, valid for surfaces whose radius of curvature is large compared with the thickness of the double layer,

$$\sigma = \sqrt{\frac{\mathbf{N}DkT}{2000\pi}} \sqrt{\sum c_i \left(e^{-z_i \frac{e\zeta}{kT}} - 1\right) + \sum c_j \left(e^{+z_j \frac{e\zeta}{kT}} - 1\right)}, \quad (4)$$

where c_i and c_j are the concentrations of the cations and anions respectively (Abramson and Müller, 1934, Müller, 1934a, Abramson, 1934a, 1934b, 1935, Moyer and Bull, 1935, Abramson and Moyer, 1936, Moyer, 1934).

It is possible to test the validity of this equation by using the results presented in figure 3 (lower). There we have data for mobilities of the same organism (*E. coli* S) suspended in acetate buffer at pH 5.5 ($\mu = 1/50$) and also in phosphate buffer at pH 6.9 ($\mu = 1/75$). In this instance, not only are the ionic strengths different but the valences as well.

Due to the action of these two factors on λ, the curves are roughly parallel but run their courses at different levels. If we may assume that (1) σ is independent of pH between pH 5.5 and 6.9, (2) the surfaces are saturated with ions in both buffers, i.e., σ is at its maximal value, (3) the specificity of the phosphate and ions is subordinate to the effects of their concentrations and valences, and (4) the radius of the particle is large compared to the thickness of the double layer (i.e., $\kappa r \gg 1$), we are in a position to calculate σ for both buffers and to expect the values to be the same.

First, let us consider assumption (1). To verify this, a pH-mobility curve for 16-hour cultures of *E. coli* S was determined in buffers of constant ionic strength ($\mu = 1/50$); as shown in table 8, mobility and consequently σ[9] are independent of pH over the range, pH 4 – pH 7. Furthermore, as will shortly be clear, both assumptions (2) and (3) are also valid. No orientation or

[9] Since μ is constant, κ is constant and $\sigma = \sigma(v)$ only (Moyer, 1934).

dependence of mobility on size or shape were ever noted, so assumption (4) is probably justified (Abramson, 1934a).

TABLE 8

Constancy of electrophoresis of E. coli S with changes in pH

Phosphate and acetate buffers at ionic strength = 0.02. Mobilities in μ/sec./v./cm.

	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.9
Mobility	0.64	0.65	0.64	0.65	0.64

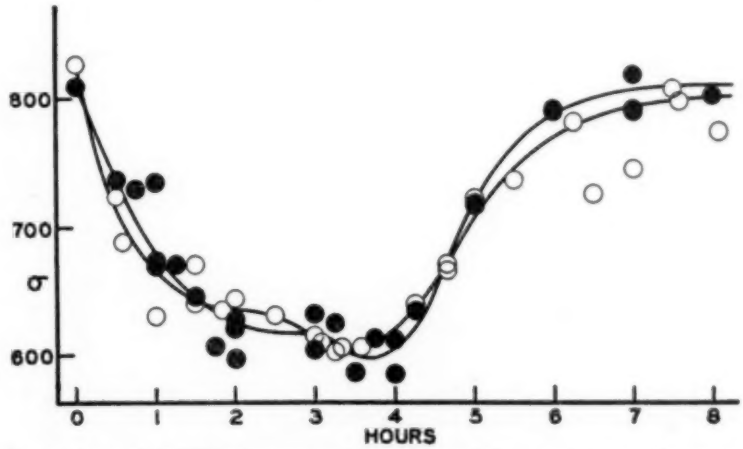


FIG. 5. CHARGE DENSITY CURVES OF E. COLI PLOTTED AGAINST AGE OF CULTURE, CALCULATED FROM DATA OBTAINED IN M/150 PHOSPHATE (OPEN CIRCLES) AND M/50 ACETATE (CLOSED CIRCLES)

Separate mean curve plotted for each set of data to show agreement. Zero hour points are mean values

σ was therefore calculated from mobilities obtained in the phosphate buffer by equations 1 and 4 while the acetate values were computed from a simpler transformation of equation 4,

$$\sigma = 2\sqrt{\frac{\mathbf{N}DkT}{2000\pi}}\sqrt{\mu}\sinh\frac{\zeta\mathbf{e}}{2\mathbf{k}T} \qquad (5)$$

completely valid when $z_i = z_j = 1$. The use of these equations has been illustrated by Moyer and Bull (1935) and Abramson and Moyer (1936).

Figure 5 shows that the two curves are superimposed, within the limits of error, as predicted (note that the scale has been much enlarged). This result not only supports the theory of Gouy but also shows the constancy of behavior of the organism.

These equations have been used with success in several fields to elucidate the nature of many surfaces (Abramson and Müller, 1934, Abramson, 1934a, 1934b, 1935, Moyer, 1934, 1935a, Moyer

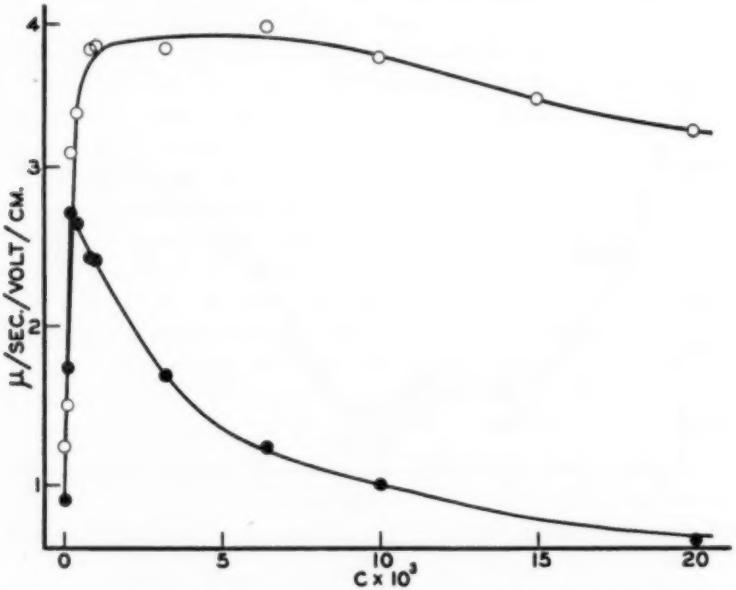


FIG. 6. MOBILITY-CONCENTRATION CURVES OF E. COLI IN ACETATE BUFFERS
Rough form: open circles. Smooth form: closed circles

and Bull, 1935, Abramson and Moyer, 1936) by following the course of the net charge density with changes in salt concentration.

To carry out such measurements in buffered media, acetate buffer initially adjusted to pH 4.6 was diluted to various acetate ion concentrations[10] (here the concentration of the sodium acetate)

[10] The pH ranged from pH 4.6 at 2×10^{-2} M acetate to pH 4.8 at 2×10^{-4} M. Below this concentration it rose to *ca.* pH 5.8 in distilled water.

and mobility-concentration curves were determined for *E. coli* R and S as shown in figure 6. It will be noted that both curves rise from low mobilities in distilled water to a maximum and slowly fall again. This is the characteristic form of the v-c curve of an "inert" surface in salts not reversing the sign of the charge (Abramson, 1934a). From these results, σ-c curves have been

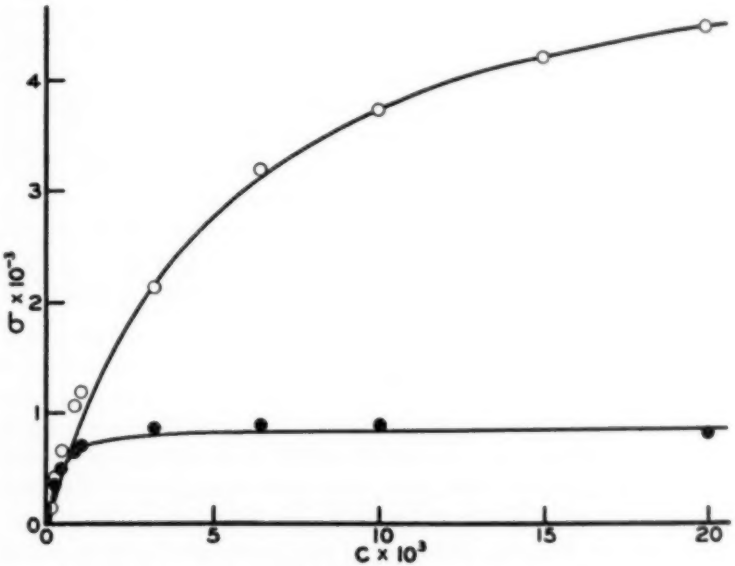


FIG. 7. CHARGE DENSITY-CONCENTRATION CURVES OF E. COLI IN ACETATE BUFFERS

Rough form: open circles. Smooth form: closed circles. Smooth curves plotted from Langmuir adsorption isotherm.

calculated, using equation 5 and correcting the total charge, σ_T, for the charge caused by the distilled water, σ_W, to obtain σ, for

$$\sigma = \sigma_T - \sigma_W, \tag{6}$$

a method first proposed by Abramson (1932).

These curves are shown in figure 7. The points are the experi-

mental values, the smooth curves have been drawn by a Langmuir adsorption isotherm of the simplest form (Hückel, 1928),

$$\sigma = \sigma_L \frac{\beta c}{1 + \beta c}, \tag{7}$$

where $\beta\sigma_L$ is the initial slope and σ_L is the limiting value of σ within the concentration range. These constants were determined by transforming equation 7 into its linear form, in which case $1/\beta\sigma_L$ is the intercept and $1/\sigma_L$ is the slope. It will be noticed that the agreement is excellent. This same equation has been fitted with success to curves of this character by Abramson and Müller (1934), Abramson (1934a, 1935) and Moyer (1934, 1935a). It is important to emphasize that electrokinetic effects are not always small in high salt concentrations. Preliminary experiments show that *E. coli* R retains a mobility of 1.7μ/sec./v./cm. (22 millivolts) in M/5 acetate. This value is within 20 per cent of that predicted by theory.

STUDIES ON SALMONELLA GALLINARUM

A few observations are available concerning the smooth form of *Salmonella gallinarum*. In confirmation of the reports of Mudd, Nugent and Bullock (1932) and Joffe and Mudd (1935) for other organisms of the intestinal group, it was found that the cells of *S. gallinarum* show no definite electrophoretic mobility in either buffer or at any age. Cells in any culture are seen migrating extremely slowly in both directions. This condition could not be changed by repeated plating. Although no changes from this practically isoelectric condition[11] were noted, it may be that the surface does change during the growth cycle, but within the range of error of our technique. It was impossible to assign a definite value to the mobilities of these organisms. The first strain had been under laboratory cultivation for many years but fresh

[11] The term "isoelectric" is used in the same sense as originally defined by Hardy, i.e., showing no motion in the electric field. The term does not imply the presence of an ionogenic surface. For a discussion of this point, see Abramson (1934a, p. 132).

strains,[12] which had been isolated from a dying chick and turkey three months before, likewise gave the same results.

DISCUSSION

It seems established that the mobility of the Cohen strain of *Escherichia coli* varies in electrophoretic mobility during the age of the culture. Although Buggs and Green report no change, they started at 6 hours, from which time we also find little if any change in v. Pedlow and Lisse made their measurements in distilled water. This medium has been repeatedly criticized (cf. Abramson, 1934a) as unsuited for comparative electrophoresis measurements because of its lack of buffering power, low value of κ, and varying ionic strength. In distilled water, the net charge density of these surfaces is low and λ is great. ζ or v, therefore, show little specificity from surface to surface. For instance, note in figure 6 the close position of the mobilities of the R and S forms in distilled water. It can be readily shown by dividing σ_W, the charge density of *E. coli* S in distilled water ($= 11.5$ e.s.u. cm^{-2}), by σ_L ($= 860$ e.s.u. cm^{-2}), that the surface in distilled water is only charged to 1.3 per cent of the maximum. This fact makes ζ extremely sensitive to accidental changes in the CO_2 and salt content of the distilled water. If enough ions are brought into the solution by incomplete washing, say, to change the net surface charge 100 per cent, v will, be increased by about 40 per cent;[13] the same number of ions ($11.5/4.77 \times 10^{-10}$) added to this surface when it is in the $\mu/75$ phosphate buffer would hardly change its mobility at all. In the light of these calculations, studies such as those by Rosenow (1933), Dozois (1936), and others might more profitably be repeated using buffers. Although in 1924 Shibley used distilled water, the changes were so great in the organisms he investigated that differences could still be detected during growth. With our *Escherichia coli* R or S, this would probably not be true.

[12] Kindly furnished by Dr. W. R. Hinshaw.

[13] It can be shown by equations 1 and 2 that $V_2/V_1 = \sqrt{2}$ (where V_1 and V_2 are mobilities before and after addition of salt, respectively), since, for low values of c, $\sigma = \sigma_i \beta c$.

Both *Escherichia coli* R and S were given the usual tests for roughness and smoothness. The R form was only one of several such variants which had arisen in our cultures but, so far as could be ascertained, the S form was the smoothest variation that this strain of *Escherichia coli* can produce. Since Joffe and Mudd report that all their smooth forms of intestinal bacteria (which included a strain of *Escherichia coli*) were isoelectric, at all pH values, it may be that our S form is slightly rough. However none of the tests gave evidence of this being so, except possibly the slightly serrate edge of cultures on extract plates, although on Endo plates edges were entire. Both R and S strains were plated frequently to preserve their homogeneity. The R forms grew as a sediment in unaerated extract broth or occasionally, when the ζ-potential was high, grew with a slight diffuseness. This need not be considered a reversion to S (Joffe and Mudd, 1935). Only the R variant with the highest potential has been investigated here. The S form was always completely diffuse in growth.

Both the 16-hour and 3-hour cells of *Escherichia coli* S are negatively charged far down the pH scale. A value for the isoelectric point is not given because Eggerth (1924) conclusively showed that *Escherichia coli* when treated with acid below pH 4 excretes substances which are reabsorbed and consequently alter the surface. We, too, find that if these organisms are washed at pH 2.5 with HCl and then re-washed at pH 6.9 with several changes of phosphate buffer, the resultant mobilities in the buffer are abnormal. The low position of the isoelectric point shows that the surface is not predominantly composed of an "ordinary" protein. It is known, however, that pepsin and silk fibroin (Abramson, 1934a) are isoelectric at very low pH values so the possibility remains that some protein of a highly acid type may be involved. Unlike the red blood cell (Abramson, 1934a), young and old cells of *Escherichia coli* S will adsorb gelatin and behave, within the limits of error, like collodion particles coated with the same preparation.

Almost no work has been done on the chemistry of *Escherichia coli* but it is probable that it is much like that of *Salmonella*

types. The chemistry of the *Salmonella* group has been well reviewed by Hornus (1935) and by Joffe and Mudd (1935). Our σ-c curves indicate that both *Escherichia coli* R and S possess "inert" surfaces. As Müller (1934b) and Abramson (1934a, 1935) have shown, the charge of surfaces may arise, in general, from two sources, (1) from adsorption, in which case the ζ-c curve rises to a maximum and declines again, or (2) from ionization (e.g., proteins), in which case there is noted a steady decline of the ζ-c curve from the lowest concentrations, with no maximum. The σ-c curves of the first type rise similarly to those in figure 7 whereas the second type possess a nearly constant charge density at all concentrations. The curves in figure 6 are quite similar to those obtained under somewhat the same conditions by both Joffe and Mudd (1935) and Northrop and de Kruif (1922). The results of previous calculations by Abramson (1934b) of σ-c curves of bacteria using data from the literature were not consistent enough to show at that time if the use of the Langmuir equation was justified for bacterial surfaces. Carbohydrate and certain lipoid surfaces are similar in behavior to these bacteria in respect to σ-c curves (Abramson, 1934a, Moyer, 1934, 1935a, 1935b, Moyer and Bull, 1935). In this connection use of the Mudd interfacial technique to investigate the wetting properties of these bacteria might prove of value (Moyer, 1935c).

In figure 8 the growth curve for aerated *Escherichia coli* S (as determined by plate counts in these experiments) has been drawn on the same graph with the mean charge density-age curve so that time values may be compared. This growth curve agrees very well with the other growth data published from this laboratory. It will be noted that the drop in charge density begins immediately and by the end of the lag period has dropped nearly to its lowest level. During the logarithmic period it remains almost constant (with a slight additional drop between 3 and 4 hours) and, as the period of negative growth acceleration sets in at 4 hours, the charge density curve slowly rises to reach a plateau value at about 8 hours. This is similar to the movements of the growth curve.

The various theories of the lag period have been well sum-

marized by Topley and Wilson (1934). Of them all, the rejuvenescence hypothesis seems the most reasonable in the light of these results. It may be that, together with the increase in size, the surfaces of the bacteria are changing to a composition more suitable for growth and division. Further work will have to be done to clarify this point. It should be emphasized that the

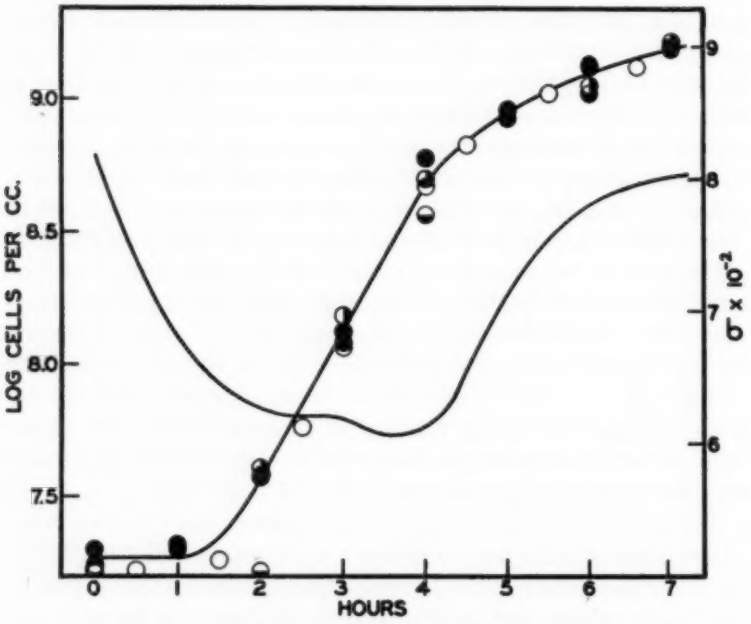


FIG. 8. GROWTH CURVE OF E. COLI S IN 1 PER CENT PEPTONE
Different circles represent plate counts made on different days. Mean σ-age curve of same organism plotted for comparison.

change in size of itself will not explain these results. It has been noted many times in cultures of about 1.5 hours of age (where both large and small cells of the same age are still present) that there was no tendency for the small cells to pass the large or *vice versa*. This is in accord with previous findings (Abramson, 1934a).

These investigations apparently support the concept of a period

of physiological youth advanced by Sherman and Albus. In connection with this, it may be worth noting that both the unaerated smooth and the aerated rough cultures, where the seed concentration was ten times that of the smooth aerated form, reach their minimal electrophoretic mobilities more rapidly, in correspondence with the phenomenon of shorter lag in the growth curve noted with higher seeding concentrations.

It would be interesting to know if the increase in size of the cell during lag is accompanied by a simple "expansion" of the surface or whether new active groups with different adsorbing potentialities are brought into the membrane as the enlargement progresses. The ease of killing by salts at this time,[14] together with the decrease in σ, might be advanced as an indication of increased permeability and hence a support of this "expansion" hypothesis. Better evidence would lie in constancy of the net charge per cell during the lag phase and after 4 hours. No accurate data are available on the size of *Escherichia coli* during small increments of time at the start of the growth cycle; consequently this point cannot be settled now.

SUMMARY

1. Electrophoretic measurements of *Escherichia coli* under carefully controlled conditions reveal that the surface factors which determine the electrokinetic potential of bacteria in mature cultures are constant during long periods of cultivation, provided rough variants are eliminated. Rough and smooth forms retain their individuality of electrophoretic behavior even in mixed culture.

2. Young cells of this organism in both the rough and smooth forms exhibit a definite decrease in electrophoretic mobility with the age of the culture, which persists from the time of seeding to about seven hours. This change has been observed in two buffers at different pH values, with and without aeration, and even in the peptone growth medium itself.

[14] Sherman and Albus (1924) report that 5 per cent NaCl, while harmless to older cultures, is markedly toxic to those in the early phases of growth. Numerous unpublished experiments of our own fully confirm these results.

3. Special precautions were taken to eliminate the possible effects of flagella, gases, adsorption from the medium and injury but the decreased mobility was always observed during the phase of the culture cycle in question.

4. Calculation of σ, the net charge density, gives support to the theories of the diffuse double layer and reveals that when the effects of differences in the thickness of the double layer are eliminated, the behavior of *Escherichia coli* is the same, within the limits of error, although suspended in different buffers at two different ionic strengths.

5. No change in the mobility of *Escherichia coli* S with pH could be noted between pH 4.0 and pH 7.0, when suspended in buffers of constant ionic strength.

6. Determination of mobility-concentration curves for the R and S types of *Escherichia coli* and calculation of the corresponding σ-c curves show that bacterial surfaces are amenable to the same physico-chemical principles used in the treatment of non-living surfaces. Possibilities as to the nature of the active groups at the surfaces of these organisms are discussed.

7. It is pointed out that the critical breaks in the charge density-age curve correspond to the breaks in the growth curve obtained for *Escherichia coli* S. Suggestions are made as to the possible significance of the lag phase and the period of physiological youth in the light of these data.

8. A few observations are presented for *Salmonella gallinarum* S. No definite electrophoretic mobility could be noted at any field strength, in agreement with Joffe and Mudd. This fact rendered it impossible to decide, with present methods, if changes similar to those in *Escherichia coli* occur in this species.

9. It is concluded that these changes are probably due to actual changes in the physical or chemical constitution of the bacterial surface during the growth cycle.

I wish to take this opportunity to thank Dr. C.-E. A. Winslow for his encouragement and helpful suggestions throughout the course of these investigations. Thanks are also due to many other friends for their critical and technical assistance, especially

Dr. H. A. Abramson, Dr. E. Huntington, Dr. H. Mestre and Dr. H. H. Walker.

REFERENCES

ABRAMSON, H. A. 1929 Jour. Gen. Physiol., **12**, 711.

ABRAMSON, H. A. 1932 Jour. Phys. Chem., **36**, 2141.

ABRAMSON, H. A. 1934a Electrokinetic Phenomena. The Chemical Catalog Co., New York.

ABRAMSON, H. A. 1934b Trans. Electrochem. Soc., **66**, 153.

ABRAMSON, H. A. 1935 Jour. Phys. Chem., **39**, 749.

ABRAMSON, H. A., AND MOYER, L. S. 1936 Jour. Gen. Physiol., **19**, 601.

ABRAMSON, H. A., AND MÜLLER, H. 1934 Cold Spring Harbor Symposia on Quantitative Biology, **1**, 29.

BAYNE-JONES, S., AND RHEES, S. H. 1929 Jour. Bact., **17**, 123.

BRAUN, H., AND SCHAEFFER, H. 1919 Berl. klin. Woch., **56**, 409.

BUGGS, C. W., AND GREEN, R. G. 1935 Jour. Bact., **30**, 453.

CHOUCROUN, N. 1934 Compt. Rend. Acad. Sci., **199**, 36.

CRAIGIE, J. 1931 Jour. Immunol., **21**, 417.

DOZOIS, K. P. 1936 Jour. Bact., **31**, 211.

EGGERTH, A. 1924 Jour. Gen. Physiol., **6**, 63.

FISHER, R. A. 1930 Statistical Methods for Research Workers. Third ed. Oliver and Boyd, London.

HEIBERG, B. 1932 Ztschr. Hyg., **114**, 425.

HORNUS, G. 1935 Rev. Immunol., **1**, 488.

HÜCKEL, E. 1928 Adsorption und Kapillarkondensation. Kolloidforsch. in Einzeldarstell., **7**, Akadem. Verlagsgesell. Leipsic.

HUNTINGTON, E. 1936 Jour. Bact., in press.

JOFFE, E. W., AND MUDD, S. 1935 Jour. Gen. Physiol., **18**, 599.

KAUFFMANN, F. 1934 Ergebn. Hyg., **15**, 219.

MARTIN, D. S. 1932 Jour. Gen. Physiol., **15**, 691.

MOONEY, G., AND WINSLOW, C.-E. A. 1935 Jour. Bact., **30**, 427.

MOYER, L. S. 1934 Biochem. Zeit., **273**, 122.

MOYER, L. S. 1935a Jour. Gen. Physiol., **18**, 749.

MOYER, L. S. 1935b Jour. Gen. Physiol., **19**, 87.

MOYER, L. S. 1935c Amer. Jour. Botany, **22**, 609.

MOYER, L. S. 1936 Jour. Bact., **31**, 531.

MOYER, L. S., AND ABRAMSON, H. A. 1936 Jour. Gen. Physiol., **19**, 727.

MOYER, L. S., AND BULL, H. B. 1935 Jour. Gen. Physiol., **19**, 239.

MUDD, S., NUGENT, R. L., AND BULLOCK, L. T. 1932 Jour. Phys. Chem., **36**, 229.

MÜLLER, H. 1934a Cold Spring Harbor Symposia on Quantitative Biology, **1**, 1.

MÜLLER, H. 1934b Cold Spring Harbor Symposia on Quantitative Biology, **1**, 34.

NORTHROP, J. H., AND DE KRUIF, P. H. 1922 Jour. Gen. Physiol., **4**, 639.

PEDLOW, J. T., AND LISSE, M. W. 1936 Jour. Bact., **31**, 235.

PILOJAN, A., KRIWORUTSCHKO, N., AND BACH, N. 1933 Kolloid Ztschr., **64**, 287.

REED, G. B., AND GARDINER, B. G. 1934 Canadian Jour. Res., **11**, 481.

REICHENBACH, H. 1911 Ztschr. Hyg., **69**, 171.

ROSENOW, E. C. 1933 Jour. Inf. Dis., **53**, 1.
SCHMIDT, C. F., JR., AND BAYNE-JONES, S. 1933 Jour. Cell. Compar. Physiol., **3**, 405.
SCHULTZ, J. H., AND RITZ, H. 1910 Zbl. Bakt., I, **54**, 283.
SHERMAN, J. M., AND ALBUS, W. R. 1923 Jour. Bact., **8**, 127.
SHERMAN, J. M., AND ALBUS, W. R. 1924 Jour. Bact., **9**, 303.
SHERMAN, J. M., AND CAMERON, G. M. 1934 Jour. Bact., **27**, 341.
SHIBLEY, G. S. 1924 Jour. Exper. Med., **40**, 453.
TOPLEY, W. W. C., AND WILSON, G. S. 1934 The Principles of Bacteriology and Immunology, **1**, 75.
WALKER, H. H. 1932 Jour. Bact., **24**, 169.
WALKER, H. H. 1936 Jour. Bact., **31**, 17.
WALKER, H. H., WINSLOW, C.-E. A., HUNTINGTON, E., AND MOONEY, M. G. 1934 Jour. Bact., **27**, 303.
WHITE, P. B. 1926 Publ. Med. Res. Council, London, No. 103.
WINSLOW, C.-E. A., FALK, I. S., AND CAULFIELD, M. F. 1923 Jour. Gen. Physiol., **6**, 177.
WINSLOW, C.-E. A., WALKER, H. H., AND SUTERMEISTER, M. 1932 Jour. Bact., **24**, 185.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTHERN CALIFORNIA-HAWAIIAN BRANCH

UNIVERSITY OF WASHINGTON, SEATTLE, WASH., JUNE 19, 1936

In conjunction with the Western Division of the American Association for the Advancement of Science

DESENSITIZATION IN THE TREATMENT OF TUBERCULOUS GUINEA PIGS. *J. Durward Thayer*, University of Washington, Seattle.

In the present paper, several antigens have been tested for their desensitizing power, and the two which proved most satisfactory have been given more extensive study.

Results. I. (1) Two bacillary antigens, one killed by ether and chloroform and the other by nitrous acid, are found to accomplish more effective desensitization when given in daily increasing doses than when administered in weekly constant doses.

(2) Bacillary antigens are unsuitable agents for desensitization because of the massive adhesions and sterile abscesses they cause upon injection.

II. An antigen, prepared by extracting tubercle bacilli with glycerol is highly specific and brings about good desensitization.

III. Tuberculin (S.M.T.) prepared by the "Sensitized Vaccine" method of Besredka, renders large doses of tuberculin non-toxic for the sensitive tuberculous guinea pig, but, at the same time, the greater part of the tuberculin desensitizing power is lost.

IV. Tuberculin (S.M.T.) treated with nitrous acid, is apparently so denatured that it no longer serves as a satisfactory desensitizing agent.

V. (1) The tuberculin, T. P. T., is a slightly better antigen than Old Tuberculin for the production of desensitization; smaller amounts being required to bring about an equal degree of desensitization.

(2) Desensitization treatment employing the tuberculins T.P.T. and P.P.D. greatly reduces the skin sensitiveness and the general systemic allergy of tuberculous guinea pigs.

(3) The gross and microscopic lesions of guinea pigs treated daily with tuberculin are less extensive than those of the control animals.

THE RAPID PRODUCTION OF TUBERCULIN ON AN INEXPENSIVE SYNTHETIC MEDIUM. *Sam Wong*, The Alice McDermott Foundation, University of Washington, Seattle.

A comparative study was made to correlate the weight of organisms to the yield of tuberculin in 4 synthetic media at the end of 6 weeks of growth, using *Mycobacterium tuberculosis*, H 37. It was found that the bulk of organisms was not so essential in the rapid production of tuberculin as was a slightly alkaline terminal pH. There was presented a glycerol-free medium which at the end of 6 weeks contained about half of the weight of organisms yielded by Long and Seibert's medium but yielded 3 times as much tuberculin.

Furthermore, the yield of tuberculin at the end of 6 weeks on this medium was about the same as on Long and Seibert's at 12 weeks. Evidence was presented to show that the yield of tuberculin on the modified Long and Seibert's medium can be increased twofold merely by readjusting the terminal acid pH of 5.8 to a slightly alkaline pH of 7.2 by means of sterile 10 per cent sodium carbonate solution and incubating for 2 more weeks. Glucose was a satisfactory substitute for glycerol in growing H37 under the conditions specified in the report. It was better as a carbon source than glycerol in the production of synthetic medium tuberculin. The tuberculoprotein obtained from the glycerol-free medium was apparently identical with those obtained from other glycerol media; comparative skin tests and systemic tests made on sensitive tuberculous guinea pigs showed no difference in reaction.

ANTIGENIC RELATIONSHIPS OF SOME TUBERCLE BACILLI AS DETERMINED BY SEROLOGICAL REACTIONS WITH THEIR NATURAL TUBERCULINS. *Russell S. Weiser*, University of Washington, Seattle.

Natural tuberculins, which are filtered saline extracts of tubercle bacilli obtained by the use of liquid air in the repeated alternate freezing and thawing of tubercle bacilli, were injected intraperitoneally into young incubator-hatched Leghorn pullets. The extracts were derived from virulent avian, human and bovine strains of tubercle bacilli. One week after a series of 11 weekly injections of antigen the birds were bled and the serum tests made for precipitin, cross precipitin and precipitin absorption reactions to all of the natural tuberculins employed. The minimum absorbing dose was determined for each serum and used for absorption studies.

The avian strain could be differentiated from the human and bovine strains by cross precipitin and precipitin absorption reactions, whereas the bovine and human strains could be differentiated from each other only by precipitin absorption reactions with their homologous tuberculins.

The results indicate that tubercle bacilli of human and bovine strains are antigenically similar although not identical and that they differ markedly in this respect from the avian strain.

BACTERIOLOGICAL PROBLEMS OF CANNING INDUSTRY. *J. R. Esty*, National Canners Research Laboratory, San Francisco.

THE THERMAL DEATH TIME OF CLOSTRIDIUM BOTULINUM SPORES AT TEMPERATURES AND pH VALUES COMMONLY ENCOUNTERED IN HOME CANNING. *W. V. Halversen and Glen L. Hays*, Bacteriology Department, University of Idaho, Moscow, Idaho.

This study presents data showing a comparison of the thermal death time at temperatures corresponding to the boiling point of water at altitudes commonly encountered in Idaho and also at temperatures encountered in the pressure cooker. These data emphasize the extreme exposures necessary to kill the spores at temperatures corresponding to the boiling point of water. It appears obvious that a steam pressure cooker would be necessary for processing vegetables at higher altitudes. In this experiment, at 90°C., which would correspond to the boiling point of water at 9000 feet altitude, the processing time for hominy would be 10 hours, corn 9¼ hours, spinach and bean 8½ hours, pears 2¼ hours, and prunes 1 hour. These exposures are

much greater than those recorded in the commercial time tables where the rule is to increase the processing time 10 per cent for each additional 500 feet above the first 1000 feet altitude. We would therefore conclude that the recommended time is inadequate.

Time in minutes to destroy spores of Clostridium botulinum at varying pH values and temperatures

	pH	90°C.	95°C.	100°C.	105°C.	110°C.	115°C.
Hominy	6.95	600	495	345	120	35	10
Corn	6.45	555	465	255	105	30	15
Spinach	5.10	510	345	225	90	20	10
String beans	5.10	510	345	225	90	20	10
Pumpkin	4.21	195	120	45	20	15	10
Pears	3.75	135	75	30	10	10	5
Prunes	3.60	60	20				

Since several outbreaks of botulism have been traced to pears, it seems logical to recommend that, at altitudes above sea level pears should be processed in the steam pressure cooker.

THE WATER CONTENT OF BACTERIAL SPORES. *B. S. Henry and C. A. Friedman*, University of Washington, Seattle.

The water content of the spores and vegetative cells of *Bacillus subtilis*, *Bacillus megatherium* and *Bacillus mycoides* was determined. Using air dried cells as a basis the moisture contents of the two types of cells were found to be almost identical. Moist weight is considered unsatisfactory as a basis for comparisons of the two types. It was concluded that differences in water content in the two types of cells is not the true explanation of the differences in heat resistance of the two forms. The possibility of a difference in the ratio of free and bound water in the two cell types is suggested.

EFFECT OF A STARVATION MEDIUM ON BACTERIAL VARIATIONS. *Victor Burke*, Washington State College.

Water containing 200 mgm. of potassium permanganate and 750 mgm. potassium hydroxide per liter was distilled from a pyrex flask. The water was immediately sterilized. Agar agar was thoroughly washed in distilled water and dried. A medium consisting of 1.5 per cent agar agar was made by the addition of the distilled water titrated to pH 7.2 and sterilized. This starvation medium was inoculated with washed cells of *Sarcina lutea*, *Staphylococcus albus*, *Staphylococcus aureus*, *Rhodococcus rosaceus*, *Escherichia coli* and *Bacillus megatherium*.

With *Sarcina lutea* about one-third of the plates showed growth in 3–5 weeks. The morphology of the colonies varied greatly—none were typical except on two plates where all that developed were typical. Nearly all extremes in colony types were noted, rough, spreading, discrete, G colonies, daughter colonies, etc. Only two pigments showed up, a lemon yellow characteristic of the stock culture and a deep orange. Pigments did not appear on the plates, only on the cultures on slants taken from the plate. The colony changes appeared temporary, the pigment permanent. On all but two plates the morphology of the cells and staining reaction varied. Some colonies consisted of Gram-negative cocci and Gram-negative bacilli. Other colonies consisted of Gram-negative bacilli. In general when colonies were typical the cells were typical. When colonies were atypical the cells were atypical. Fermentation tests indicated some variations from normal.

Attempts to bring about reversion have so far failed. Starting with a

colony consisting wholly of bacilli, no cocci ever appeared on successive slants. However, reversion may occur in some other medium or following some other treatment. If reversion never occurs the bacillus represents a mutation, and if the Gram-negative cocci never revert to the Gram-positive possibly they also represent a mutation.

Comparable results with minor differences were obtained with *Staphylococcus aureus*. A Gram-negative yellow-pigment-producing bacillus that did not revert to the original type was produced at will. The colonies were all on the surface. Some had a faint lavender shade.

Escherichia coli developed atypical colonies and a yellow pigment. The pigment was retained on potato extract agar. The colonies were all surface colonies. *Bacillus megatherium* grew with atypical colony formation. Other changes have not been determined. Some organisms failed to grow.

Since double-distilled water is toxic we should consider that single-distilled water is slightly toxic even though growth occurs. Therefore, the variations arising may be due to either of two causes, starvation or toxicity of the water. Distillation leads to a reduction of salts as well as of organic matter. The addition of a small amount of potato juice, containing organic matter and salts, causes the toxicity to disappear.

The change in colony formation on the starvation medium is to be expected since growth is very slow. The other changes are more permanent and of greater significance. The biophysics involved in turning a coccus into a bacillus is obscure. Such a change might conceivably result from the weakening of the cell wall. The fact that the change in staining is toward the Gram-negative may offer support to this view. We have experiments under way to determine whether there is a change in the permeability of the cell wall. If the Gram reaction depends on the permeability of the cell wall as I believe then there must be such a change.

CHEMOPROPHYLAXIS OF POLIOMYELITIS. *E. W. Schultz and L. P. Gebhardt*, Stanford University.

RELATIONSHIP BETWEEN NASAL AND HUMORAL ANTIPOLIOMYELITIC SUBSTANCES. *B. F. Howitt*, Hooper Foundation, San Francisco, California.

Based on the work of Amoss and Taylor in 1917 dealing with presence of neutralizing substances in the nasal washings of different individuals, a comparison has been made between the humoral and nasal antipoliomyelitic substances in a group of 61 individuals. This group was divided into 2 groups of about 30 each, one half adults over 25 years of age and the other children varying in age from 2 to 15 years. Both adults and children were subdivided into 3 groups of 10 each, those with apparently normal and with abnormal nasal mucosa, and a third series representing those recovered from poliomyelitis after varying periods of time.

In vitro neutralization tests were positive on 36 (59 per cent) of the serums and 14 (22.9 per cent) of the filtered nasal washings from a total of the 61 individuals examined. No particular correlation could be noted between the presence of positive serums and positive nasal filtrates, although more of the latter occurred among so-called normal adults than among the recovered poliomyelitic cases. Nine individuals gave positive tests in both serums and nasal filtrates, 5 showed

antiviral washings and negative serums, while in 27 the serums were positive and the filtrates negative. About an equal number of positive filtrates were found among those with abnormal nasal conditions as among those with normal. Serums of 71.3 per cent of the normal adults were antiviral as compared to 60 per cent from those recovered from poliomyelitis, while serums from 55 per cent of normal children were positive as compared to 40 per cent from the group of recovered poliomyelitic cases.

Concentration of nasal filtrates by the Flosdorf and Mudd lyophile apparatus was of value in securing more positive neutralization tests. No antiviral substances were found in the nasal washings of 5 immunized monkeys, taken at different stages of immunity. Their tissue immunity failed to run parallel with the humoral response, since 3 animals possessed neutralizing antibodies in the serum without developing resistance to intracerebral inoculation of virus.

ANTIBODY AND IMMUNITY IN POLIOMYELITIS. *J. F. Kessel, R. T. Fisk and F. Stimpert*, University of Southern California.

COMPARATIVE STUDY OF TWO STRAINS OF ROCKY MOUNTAIN SPOTTED FEVER VIRUS WITH SPECIAL REFERENCE TO THE WEIL-FELIX REACTION. *G. E. Davis*, U. S. P. H. S. Rocky Mountain Spotted Fever Laboratory.

A STUDY OF SEVERAL FACTORS AFFECTING ALCOHOL FORMATION BY SACCHAROMYCES ELLIPSOIDEUS. *Leonore Hall and W. V. Cruess*, University of California.

The so-called syruped method of fermentation first described by Cruess, Brown and Flossfeder (Jour. Ind. Eng. Chem., **8**, 1124, 1916) was used in securing the data reported. Of the factors studied temperature proved the most important; at 7–20°C. approximately 16.5 per cent alcohol by volume was attained; at 25°C., 13.8 per cent and at 36°C., only 6.4 per cent.

Grape concentrate proved more suitable than other materials used as substrates for pure sugars compared in respect to maximum alcohol attained.

Of the various fruit and vegetable juices compared, tomato and grapefruit juices attained the highest alcohol content.

Various yeast foods and vitamin concentrates were added at the time of syruping in several experiments; of these di-ammonium phosphate gave the greatest increase in final alcohol content. Vitamin C appeared to have a positive effect also. Several small additions of grape concentrate gave higher final alcohol content than a single large addition.

The evidence indicates that some substance or substances other than sugar alone also influences the remarkably high alcohol yields observed in this method of fermentation.

HEMOLYTIC STREPTOCOCCI IN MARKET MILK. *J. E. Simmons*, Oregon State College.

One hundred and one cultures, which produced definite evidences of hemolysis, were isolated from samples of market milk. Forty-one of these proved to be streptococci occurring in chains having more than ten cells. None of these cultures fermented inulin or raffinose while all were able to ferment glucose, lactose and sucrose. The ability to ferment salicin and mannitol was variable. Thirteen of the cultures gave complete test tube hemolysis in two hours at 37°C. All cultures produced a pH of 4.6–5.0 in glucose

colony consisting wholly of bacilli, no cocci ever appeared on successive slants. However, reversion may occur in some other medium or following some other treatment. If reversion never occurs the bacillus represents a mutation, and if the Gram-negative cocci never revert to the Gram-positive possibly they also represent a mutation.

Comparable results with minor differences were obtained with *Staphylococcus aureus*. A Gram-negative yellow-pigment-producing bacillus that did not revert to the original type was produced at will. The colonies were all on the surface. Some had a faint lavender shade.

Escherichia coli developed atypical colonies and a yellow pigment. The pigment was retained on potato extract agar. The colonies were all surface colonies. *Bacillus megatherium* grew with atypical colony formation. Other changes have not been determined. Some organisms failed to grow.

Since double-distilled water is toxic we should consider that single-distilled water is slightly toxic even though growth occurs. Therefore, the variations arising may be due to either of two causes, starvation or toxicity of the water. Distillation leads to a reduction of salts as well as of organic matter. The addition of a small amount of potato juice, containing organic matter and salts, causes the toxicity to disappear.

The change in colony formation on the starvation medium is to be expected since growth is very slow. The other changes are more permanent and of greater significance. The biophysics involved in turning a coccus into a bacillus is obscure. Such a change might conceivably result from the weakening of the cell wall. The fact that the change in staining is toward the Gram-negative may offer support to this view. We have experiments under way to determine whether there is a change in the permeability of the cell wall. If the Gram reaction depends on the permeability of the cell wall as I believe then there must be such a change.

CHEMOPROPHYLAXIS OF POLIOMYELITIS. *E. W. Schultz and L. P. Gebhardt*, Stanford University.

RELATIONSHIP BETWEEN NASAL AND HUMORAL ANTIPOLIOMYELITIC SUBSTANCES. *B. F. Howitt*, Hooper Foundation, San Francisco, California.

Based on the work of Amoss and Taylor in 1917 dealing with presence of neutralizing substances in the nasal washings of different individuals, a comparison has been made between the humoral and nasal antipoliomyelitic substances in a group of 61 individuals. This group was divided into 2 groups of about 30 each, one half adults over 25 years of age and the other children varying in age from 2 to 15 years. Both adults and children were subdivided into 3 groups of 10 each, those with apparently normal and with abnormal nasal mucosa, and a third series representing those recovered from poliomyelitis after varying periods of time.

In vitro neutralization tests were positive on 36 (59 per cent) of the serums and 14 (22.9 per cent) of the filtered nasal washings from a total of the 61 individuals examined. No particular correlation could be noted between the presence of positive serums and positive nasal filtrates, although more of the latter occurred among so-called normal adults than among the recovered poliomyelitic cases. Nine individuals gave positive tests in both serums and nasal filtrates, 5 showed

antiviral washings and negative serums, while in 27 the serums were positive and the filtrates negative. About an equal number of positive filtrates were found among those with abnormal nasal conditions as among those with normal. Serums of 71.3 per cent of the normal adults were antiviral as compared to 60 per cent from those recovered from poliomyelitis, while serums from 55 per cent of normal children were positive as compared to 40 per cent from the group of recovered poliomyelitic cases.

Concentration of nasal filtrates by the Flosdorf and Mudd lyophile apparatus was of value in securing more positive neutralization tests. No antiviral substances were found in the nasal washings of 5 immunized monkeys, taken at different stages of immunity. Their tissue immunity failed to run parallel with the humoral response, since 3 animals possessed neutralizing antibodies in the serum without developing resistance to intracerebral inoculation of virus.

ANTIBODY AND IMMUNITY IN POLIOMYELITIS. *J. F. Kessel, R. T. Fisk and F. Stimpert*, University of Southern California.

COMPARATIVE STUDY OF TWO STRAINS OF ROCKY MOUNTAIN SPOTTED FEVER VIRUS WITH SPECIAL REFERENCE TO THE WEIL-FELIX REACTION. *G. E. Davis*, U. S. P. H. S. Rocky Mountain Spotted Fever Laboratory.

A STUDY OF SEVERAL FACTORS AFFECTING ALCOHOL FORMATION BY SACCHAROMYCES ELLIPSOIDEUS. *Leonora Hohl and W. V. Cruess*, University of California.

The so-called syruped method of fermentation first described by Cruess, Brown and Flossfeder (Jour. Ind. Eng. Chem., **8**, 1124, 1916) was used in securing the data reported. Of the factors studied temperature proved the most important; at 7-20°C. approximately 16.5 per cent alcohol by volume was attained; at 25°C., 13.8 per cent and at 36°C., only 6.4 per cent.

Grape concentrate proved more suitable than other materials used as substrates for pure sugars compared in respect to maximum alcohol attained.

Of the various fruit and vegetable juices compared, tomato and grapefruit juices attained the highest alcohol content.

Various yeast foods and vitamin concentrates were added at the time of syruping in several experiments; of these di-ammonium phosphate gave the greatest increase in final alcohol content. Vitamin C appeared to have a positive effect also. Several small additions of grape concentrate gave higher final alcohol content than a single large addition.

The evidence indicates that some substance or substances other than sugar alone also influences the remarkably high alcohol yields observed in this method of fermentation.

HEMOLYTIC STREPTOCOCCI IN MARKET MILK. *J. E. Simmons*, Oregon State College.

One hundred and one cultures, which produced definite evidences of hemolysis, were isolated from samples of market milk. Forty-one of these proved to be streptococci occurring in chains having more than ten cells. None of these cultures fermented inulin or raffinose while all were able to ferment glucose, lactose and sucrose. The ability to ferment salicin and mannitol was variable. Thirteen of the cultures gave complete test tube hemolysis in two hours at 37°C. All cultures produced a pH of 4.6-5.0 in glucose

broth. All hydrolyzed sodium hippurate and none showed any capsules. Most of the organisms could be placed in two large groups having the general characteristics of *Streptococcus mastitidis* and *Streptococcus infrequens*. Serological and bacteriophage studies are being carried out to study further the relationship of these streptococci.

GERMICIDAL PROPERTIES OF CHRONIC COMPOUNDS, *D. B. Charlton*, Oregon State College.

OCCURRENCE AND SIGNIFICANCE OF LACTOBACILLUS THERMOPHILUS IN PASTEURIZED MILK. *D. B. Charlton*, Oregon State College.

MICROÖRGANISMS AND PHYTAMINS IN RELATION TO PLANT GROWTH. *W. B. Bollen*, Oregon State College.

AGGLUTINATIVE ACTION OF NORMAL HUMAN AND ANIMAL SERUMS ON THE PARADYSENTERY BACILLI. *H. J. Sears, Lillian Schwichtenberg and Marian Schwichtenberg*, Oregon Medical School.

ANAEROBIC BACTERIA ASSOCIATED WITH EXPERIMENTAL PULMONARY ABSCESS. *Charles Weiss*, Mount Zion Research Laboratories, San Francisco.

METHODS OF CHEMICAL ANALYSIS OF STAPHYLOCOCCUS AUREUS. *Rachel E. Hoffstadt and Wesley M. Clark*, University of Washington.

Since the dissociation in this laboratory (1932) of *Staphylococcus aureus* into eleven rough and one G or gonidial strain, three studies have been undertaken in order to elucidate the organisms' antigenic structure:

First, an agglutination study showed that each has a distinct antigenic mosaic.

Second, non-antigenic soluble specific substances, polysaccharide in nature, were isolated from the smooth and a rough variant and were found to differ widely with regard to their precipitin reactions both when adsorbed and unadsorbed on collodion.

Third, fractionation of the proteins of the smooth and a rough strain has been completed. One hundred grams of each organism were fractionated at different pH values. The purified and dried materials are being analyzed (1) chemically to identify the proteins, (2) serologically against the whole organisms and against sera prepared with the smooth and rough polysaccharide substances.

It is hoped to correlate differences found in chemical structure with antigenic and biochemical changes observed during dissociation.

BACTERIOPHAGE IN THE RHIZOBIA. *H. Katznelson*, Washington State College.

BACTERIAL FIBRINOLYSINS. *R. R. Madison and W. H. Manwaring*, Stanford University.

INFLUENCE OF PEPTONE CONCENTRATION ON THE METABOLIC ACTIVITIES OF ESCHERICHIA COLI. *C. E. Clifton*, Department of Bacteriology and Experimental Pathology, Stanford University, California.

The rates of oxygen consumption and of carbon dioxide production were determined in cultures of *Escherichia coli* and related organisms at different periods of growth in 1.0, 5.0 and 10.0 per cent peptone solutions. Evidence is presented which indicates that the maximum metabolic rate per cell observed late in the lag period of growth is due to increased cell size and to a high concentration gradient of foodstuffs between the cells and their

environment during this period. The observed decrease in the rate of metabolic activity per cell observed as the age of the cultures further increases appears to be primarily controlled by the decreased concentration of foodstuff available per cell, the same factor apparently determining the maximum population developed in a culture.

LONGEVITY OF TYPHOID BACILLI IN THE SOIL. *P. J. Beard*, Stanford University.

STUDIES ON HODGKINS DISEASE. *B. J. Olson, H. Donlop and M. Manson*, University of Minnesota.

STUDIES ON THE ANTIBODY RESPONSE IN INFECTIOUS MONONUCLEOSIS. *Sidney Raffel and G. Howard Bailey*, Johns Hopkins University. (Introduced by P. J. Beard.)

OBSERVATIONS ON MELITENSIS INFECTIONS IN THE SOUTHWEST. *K. F. Meyer*, George William, Hooper Foundation.

PHYSICAL EFFECTS OF AGARS USED IN CULTURE MEDIA. *C. S. Mudge*, Division of Dairy Industry, University of California.

THE EFFECTS OF 2-4 DINITROPHENOL ON THE GROWTH AND RATE OF METABOLISM OF YEAST. *A. Martin*, Stanford University.

METABOLISM OF VARIOUS TYPES OF SUGARS BY S AND R FORMS OF PNEUMOCOCCUS[1]

PHILIP FINKLE

Division of Laboratories, the Mt. Sinai Hospital, New York

Received for publication May 23, 1936

In a previous communication (Finkle, 1931) the writer reported observations upon the glucose metabolism of both S and R forms of *Pneumococcus*, Types I, II, and III. The intensity of respiration, or oxidation of glucose, was found to vary with the type. On conversion of the smooth to the rough forms, the respiratory capacities of Types I, and III changed, the former type showing an increase of 110 per cent, the latter a decrease of 45 per cent. The anaerobic glycolysis (fermentation) was approximately the same for all three groups. There were changes in the capacity to effect aerobic glycolysis in Types I, and III on conversion to R forms.

It was considered of interest to ascertain whether there are any differences in the metabolism of various types of sugars by *Pneumococcus*. Studies were undertaken of the oxidation and fermentation by both S and R forms of *Pneumococcus* of the following sugars: (*a*) The monosaccharides—glucose, galactose, and fructose; (*b*) the disaccharides—sucrose and maltose, and (*c*) the polysaccharide—inulin.

METHODS

For measuring respiration and fermentation the method described by Warburg (1923) and used by the writer (1931) in the studies upon the glucose metabolism of *Pneumococcus* was employed.

The pneumococci were grown at 37.5°C. for ten hours upon beef-heart broth of pH 7.8 in flasks containing about 200 cc. of media.

[1] Aided by a grant from Mr. Nelson I. Asiel.

The cultures were centrifuged and washed with Ringer solution, the contents of either one or two flasks being used, depending upon the heaviness of growth. The washed bacteria were resuspended in 5 to 10 cc. of Ringer solution, one suspension being used for each experiment.

Strains from different sources were used, without variation in results.

The oxygen consumption and glycolysis were measured in vessels containing Ringer solution to which was added $NaHCO_3$, in such concentration as to give a final pH of approximately 7.8. The sugar concentration was 0.1 per cent.

For each experiment seven vessels were run in parallel, one vessel (containing no bacteria) being used for barometric control. To each vessel was added the same quantity of Ringer solution, $NaHCO_3$, and the appropriate sugar. Equal quantities of the same suspension of bacteria were then added to each of the 6 vessels. In every experiment the contents of each of the 6 vessels were identical both as to milieu and quantity of bacteria, except that each contained a different one of the 6 sugars studied.

The oxygen consumption was measured in an atmosphere of air.

By the method here employed, the acid production was determined manometrically by measuring the CO_2 produced by the interaction of the acid with the $NaHCO_3$. Fermentation was measured in an atmosphere of 95 per cent N_2 and 5 per cent CO_2.

In earlier studies the writer (1931) measured the rate of the respiratory and glycolytic metabolism per milligram of dried weight of pneumococci. The work recorded here is concerned with a quantitative comparison of the metabolic activities of a given quantity of a uniform emulsion of pneumococci in the presence of various sugars. The weight or number of bacteria is irrelevant, since it is the same for all six sugars studied.

STUDIES ON RESPIRATION OF S (SMOOTH) AND R (ROUGH) FORMS OF PNEUMOCOCCUS

(*a*) *Respiration of Pneumococcus Types I, II, and III* (*S forms*)

From figure 1, which represents, diagramatically, the results of the studies, it is seen that in parallel experiments, the one variant

being the sugar employed, the oxygen consumption of *Pneumococcus* Types I, II, and III varies with the sugar used.

Pneumococcus Type I

For *Pneumococcus* Type I the respiration is greatest in the presence of fructose, decreasing for the other sugars in the following order: sucrose, maltose, glucose, and galactose—being lowest for galactose. The oxidation of galactose is approximately 50 per cent as great as that of fructose.

It is interesting to note that *Pneumococcus* Type I does not oxidize inulin.

Pneumococcus Type II

Pneumococcus Type II also oxidizes fructose at a much greater rate than it does any of the other sugars employed. In the presence of fructose about 220 c.mm. of oxygen are consumed, while there is an oxygen consumption of only 110 c.mm. in the presence of glucose. The rate of respiration decreases for the other sugars in the following order: glucose, galactose, maltose, and sucrose. For the latter two sugars the rate of oxygen consumption is practically equal, about 70 c.mm., being 32 per cent of the respiration in the presence of fructose.

There is no respiration of *Pneumococcus* Type II in the presence of inulin.

Pneumococcus Type III

The respiration of *Pneumococcus* Type III takes place at a greater rate in the presence of fructose than with the other sugars studied, the oxygen consumption being about 180 c.mm. For the other sugars the rates decrease in the following order: maltose, glucose, galactose, sucrose. The respiration in the presence of the latter sugar is about 55 per cent of that in the presence of fructose.

No oxygen consumption takes place in the presence of inulin.

On comparing the rates of respiration of *Pneumococcus* Types I, II, and III with one another, one finds (figure 1) that for all three types the maximum oxygen consumption occurs in the presence of fructose, while the minimum oxygen consumption in the case

of Type I is with either glucose or galactose, and for the other two types with either sucrose or galactose.

(*b*) *Respiration of R forms of pneumococcus derived from Types I, II, and III (S forms)*

From figure 1 it is seen that, as is the case with the S forms from which they are derived, the respiration of R forms of *Pneumococcus* varies with the different sugars used.

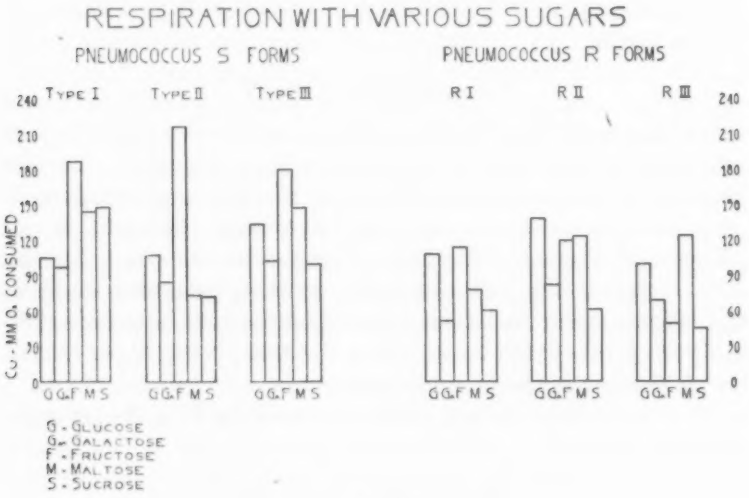


FIG. 1

Pneumococcus RI[2]

Figure 1 shows that in the case of *Pneumococcus* RI the maximum respiration takes place in the presence of fructose, with a practically equal oxygen consumption in the presence of glucose, however. The minimum oxygen consumption occurs with galactose, being about 47 per cent of the rate with fructose.

There is no respiration when inulin is used.

[2] The terms RI, RII, and RIII as used here indicate the rough forms of *Pneumococcus* derived from S Strains of *Pneumococcus* Types I, II, and III respectively.

Pneumococcus RII

The respiratory activity of *Pneumococcus* RII is at a maximum in the presence of glucose. The oxygen consumption in the presence of fructose and maltose occurs practically at the same rate as in the presence of glucose. The minimum respiration occurs when sucrose is utilized, being about 45 per cent of the maximum.

No oxygen consumption was found to take place in the presence of inulin.

Pneumococcus RIII

In the case of *Pneumococcus* RIII the greatest rate of oxygen consumption occurs when maltose is used, the rate with glucose being less. The minimum respiration occurs in the presence of either sucrose or fructose, being about 38 per cent of the maximum.

No oxygen consumption takes place when inulin is used.

It is of interest to compare (fig. 1) the respiratory activities of the R forms with one another and with the S forms from which they are derived.

While it has been shown that the maximum respiration for Types I, II, and III occurs in the presence of fructose, this is not the case with their R forms. For RI fructose, and for RII fructose or maltose are practically as effective as is glucose.

The respiratory capacity of RI is the same for glucose and for fructose, while in the case of the S form from which it is derived the respiration in the presence of fructose is much higher than with glucose. When Type I is converted to the R form, there occurs a relative increase in the capacity to oxidize glucose (as shown in a previous communication (Finkle, 1931)). There is apparently not a corresponding increase in the capacity to oxidize fructose.

While in the case of *Pneumococcus* Type II the oxidation of fructose occurs at about double the rate of oxidation of glucose, this is not the case when the organism is converted to the R form. In the latter case the oxidation of glucose and fructose occurs at about the same rate. In a previous communication (Finkle, 1931)

it was shown that there is relatively little change in the rate of glucose oxidation when *Pneumococcus* Type II is converted to the R form. The experiments presented here indicate, therefore, that there is a considerable relative decrease in the capacity to oxidize fructose when the conversion occurs.

When *Pneumococcus* Type III is converted to the R form, there is an almost 50 per cent decrease in its capacity to oxidize glucose (Finkle, 1931). The rate of fructose oxidation for Type III is

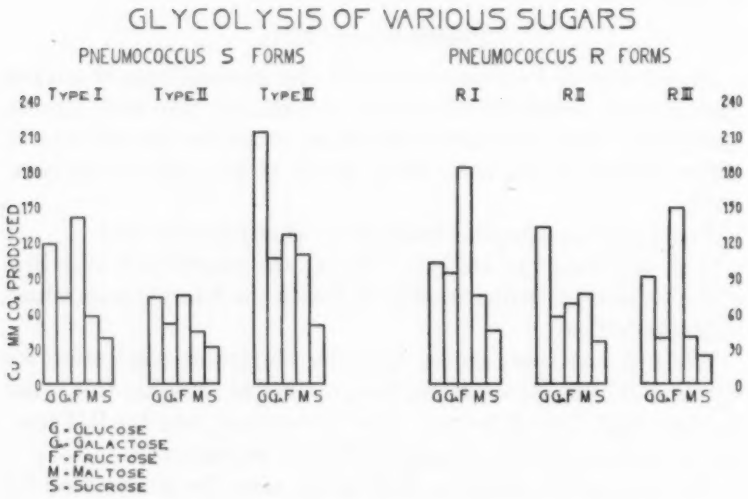


FIG. 2

greater than that of the other sugars studied. On conversion to the R form, the respiration in the presence of fructose falls to about 45 per cent of the level for glucose. There is evidently a relatively greater diminution in its capacity to oxidize fructose than in its capacity to oxidize glucose, when *Pneumococcus* Type III is converted to the R form.

It is of interest to note that neither the S nor the R forms studied, have, under the conditions of the experiments, the capacity to oxidize inulin.

STUDIES IN GLYCOLYSIS OF S AND R FORMS OF PNEUMOCOCCUS

(a) *Glycolysis of Pneumococcus Types I, II, and III*

The studies upon the glycolytic activities of *Pneumococcus* Types I, II, and III have shown that these organisms ferment the various sugars employed at varying rates (fig. 2).

Pneumococcus Type I

From figure 2 one sees that *Pneumococcus* Type I glycolyses glucose at about the same rate as it does fructose. It glycolyzes galactose, maltose, and sucrose at decreasing rates, in the order given. The glycolysis of sucrose is about 29 per cent of the maximum. There is no fermentation in inulin.

Pneumococcus Type II

Pneumococcus Type II effects maximum glycolysis in the presence of either glucose or fructose. The glycolysis decreases in the presence of galactose, maltose, and sucrose, in the order given. The glycolysis of sucrose is about 43 per cent as great as that of either glucose or fructose. There is no glycolysis of inulin.

Pneumococcus Type III

In the case of *Pneumococcus* Type III the maximum glycolysis occurs when glucose is used. The rate of glycolysis of the other sugars decreases in the following order: fructose, maltose, galactose, sucrose. The rate of fructose glycolysis is about 50 per cent that of glucose. Sucrose is glycolyzed at 24 per cent of the rate at which maximum glycolysis occurs. There is no glycolysis of inulin.

If one compares (fig. 2) the glycolysis of *Pneumococcus* Type I, II, and III in the presence of the various sugars, one finds that Types I and II are similar in this respect. The maximum glycolysis takes place in the presence of glucose or fructose, the rate being about equal for both sugars. For Type III, glycolysis is at its maximum in the presence of glucose; while glycolysis of fructose is only 50 per cent as great as that of glucose. The minimum rate of glycolysis for all three types occurs in the presence of sucrose.

It is of interest to note that there is no glycolysis of inulin by any one of the three types of *Pneumococcus* studied.

(*b*) *Glycolysis of R forms of Pneumococcus derived from Types I, II, and III*

Pneumococcus RI

From figure 2 it is seen that the maximum glycolysis of RI *Pneumococcus* occurs in the presence of fructose, and that the minimum occurs when sucrose is used. The glycolysis of glucose is 55 per cent that of fructose. Glycolysis of galactose and maltose occurs at about the same rate—somewhat less rapidly than that of glucose.

Pneumococcus RII

For *Pneumococcus* RII, the maximum glycolysis occurs in the presence of glucose, the rate decreasing with maltose, galactose, fructose, and sucrose—in the order given. The glycolysis of maltose is 58 per cent as great as that of glucose. There is no glycolysis of inulin.

Pneumococcus RIII

Pneumococcus RIII glycolyses fructose to a greater extent than it does other sugars. Next in order is glucose, whose rate of glycolysis is 60 per cent that of fructose. Sucrose is glycolysed at a minimum rate, 20 per cent as rapidly as fructose. There is no glycolysis of inulin.

It is of interest to compare the glycolytic activities of Pneumococcus RI, RII, and RIII among themselves and with the smooth forms from which they are derived (fig. 2).

The maximum glycolysis for RI and RIII occurs in the presence of fructose, while for RII the maximum takes place when glucose is utilized. For all three forms, the minimum glycolytic activity occurs when sucrose is used.

The R forms of *Pneumococcus* Types I, II, and III have a capacity to glycolyse glucose at a rate about 25 per cent greater than that exhibited by the S forms (Finkle, 1931). On converting Type I to RI, the capacity to glycolyse fructose is enhanced to a

relatively greater degree than in the case of glucose, so that fructose glycolysis becomes 80 per cent more rapid than that of glucose. A similar change in glycolytic activity occurs when Type III is converted to RIII.

On conversion of Type II to its R form there does not take place an increase in capacity to glycolyse fructose, but rather a decrease in this capacity, the glycolysis of fructose being only 54 per cent that of glucose.

In all three types, both S and R forms, the capacity to glycolyse sucrose is lowest.

INULIN

Under the conditions of these experiments, not any of the three types nor their R forms, have the capacity to glycolyse inulin.

In order to test our cultures, as well as the inulin, the following experiment was repeated several times. Into each of 2 tubes of Hiss inulin-serum-water medium (containing litmus indicator) was seeded 0.5 cc. from a pneumococcus culture. The culture was then centrifuged, washed with Ringer, and then, by addition of Ringer solution, was brought up to the same volume as before centrifugation. Into each of 2 other tubes of the Hiss inulin-serum-water medium was seeded 0.5 cc. of the suspension of washed pneumococci in Ringer solution. After incubation for twenty-four hours, all four tubes showed evidence of acid production, as indicated by change in the color of the medium from blue to red and by coagulation of the medium. The same washed cultures, when studied for respiration and fermentation, failed to utilize inulin, although metabolizing each of the other sugars employed.

DISCUSSION

For the strains studied, the S and R forms of *Pneumococcus* oxidize and glycolyse various types of sugars—glucose, galactose, fructose, maltose, and sucrose—at varying rates of speed. Among these sugars, fructose apparently affords the optimum conditions for oxidation in the case of all three S types. For Type III, however, either glucose or maltose is practically as effective as

fructose. Considering these organisms as a group, galactose is the sugar least effective for stimulating the respiratory activity of Types I, II, or III (S forms).

When one considers the glycolytic metabolism of the S forms, one finds that glucose and fructose are equally effective as the medium for maximum glycolysis of Types I and II. In the case of Type III, however, the greatest glycolytic activity occurs with glucose, fructose being only 78 per cent as effective.

These experiments suggest that for optimum carbohydrate metabolism of *Pneumococcus* Types I and II, fructose might be the medium of choice, rather than glucose, since both maximum respiration as well as maximum glycolysis takes place with the former sugar. For Type III, however, glucose would seem to remain the sugar of choice, glycolysis being at the maximum with glucose, and respiration being practically at the same rate in the presence of the latter sugar as with fructose.

On conversion of Types I, II, and III to their respective R forms, there apparently takes place a change in their relative capacities to oxidize sugars. This change seems to be manifested most strikingly in a relative diminution in the capacity to oxidize fructose, this decrease being most marked in the case of Types II and III.

There is also a change, on conversion of the S forms to R forms, in their relative capacities to effect glycolysis.

For Types I and III there is a relative increase in the capacity to glycolyse fructose. For Type II, however, there is a relative decrease in fructose glycolysis, so that glycolysis in glucose is at a maximum.

Apparently, while changes from the S forms to their respective R forms results in a relative diminution in capacity to oxidize fructose, there is at the same time, except for Type II, a relative increase in the capacity to glycolyse this sugar.

It is of considerable interest that in these experiments inulin is neither oxidized nor fermented by either the S or R forms of any of the three types of pneumococci. These startling results are at variance with the accepted belief that pneumococci produce acid from inulin, as demonstrated by the acidification of such a

medium as the Hiss inulin-serum-water media by cultures of pneumococci.

A possible explanation for this discrepancy suggests itself. It may be that the capacity of pneumococci to metabolize inulin is associated with cell growth and division, and that this metabolic function is manifested in growing cultures only, and not in suspensions of washed cells in Ringer solution where no growth can take place.

SUMMARY

1. Studies are recorded upon respiration and glycolysis by both virulent and non-virulent forms of *Pneumococcus* of the following sugars—glucose, fructose, galactose, maltose, sucrose, and inulin.

2. It was found that under the conditions of these experiments the pneumococci neither oxidize nor ferment inulin.

3. Fructose is oxidized by the S forms of *Pneumococcus* at a more rapid rate than any of the other sugars studied.

4. On conversion of virulent to non-virulent forms, the rate of fructose oxidation becomes relatively diminished, so that for RI and RII, glucose and fructose oxidation occur at about equal rates.

5. In the case of RIII, the speed of maltose oxidation is greater than that of the other sugars.

6. The rate of glycolysis of fructose is greatest for Types I and II, though glucose fermentation occurs at almost the same rate.

7. For Type III, the rate of glycolysis of glucose is more rapid than that of the other sugars.

8. In the case of RI and RIII the rate of fructose glycolysis is greater than that of the other sugars.

9. In the case of RII the speed of glucose glycolysis is greater than that of the other sugars.

REFERENCES

FINKLE, P. 1931 Jour. Exper. Med., **53**, 661.
WARBURG, O. 1923 Biochem. Z., **142**, 317.

GROWTH AND FERMENTATION OF BACTERIA NEAR THEIR MINIMUM TEMPERATURE

MILTON J. FOTER AND OTTO RAHN

Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, New York

Received for publication May 25, 1936

The object of this paper was to study the minimum temperature of fermentation separately from that for growth, and to explain, if possible, the cause for a minimum temperature. Since both these life functions are chemical reactions, they should continue, though at a greatly reduced speed, until the medium freezes solid. This is certainly not always the case with the growth of bacteria; most of them completely cease to grow at temperatures 5 to 10 or more degrees above the freezing point.

EXPERIMENTAL APPROACH

A first set of experiments was carried out with the technique used by Rahn (1932 p. 134) using *Streptococcus lactis*, *S. fecalis*, *S. glycerinaceus*, *S. cremoris*, *S. liquefaciens*, *S. mastitidis* and *Lactobacillus acidophilus*. The bacteria were cultivated at 30°C. in a heavily buffered lactose broth (for the *Lactobacillus*, tomato juice was added). After twenty-four to thirty-six hours, the cultures were neutralized and centrifuged. The bacterial sediment, after being cooled to the desired temperature, was suspended in sterile milk of the same temperature.[1] This series demonstrated the influence of low temperature upon fermentation, independent of growth. Some of the results are given in figure 1.

[1] It was intended to make the inoculum so large that no multiplication would take place, but with *Lactobacillus* and *S. glycerinaceus*, this aim was not accomplished as the frequent plate counts revealed. The inoculum was, in million cells per cubic centimeter, with *Lactobacillus* 6, with *S. glycerinaceus* 18, with *S. lactis* 250, with *S. fecalis* 400 and with *S. liquefaciens* 500. The sigmoid curves of *S. glycerinaceus* show plainly that the number of cells slowly increased.

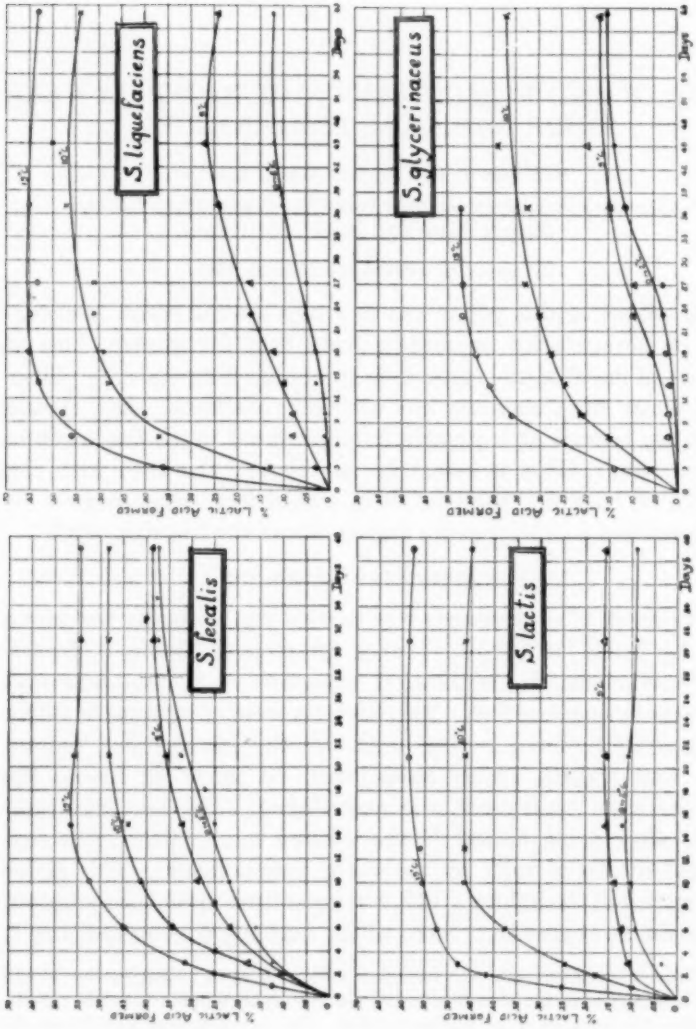


FIG. 1. DEVELOPMENT OF LACTIC ACID IN MILK BY LARGE NUMBERS OF CELLS OF STREPTOCOCCI AT LOW TEMPERATURES

A second series was carried out by inoculating sterile milk with small amounts of the same bacteria, and studying their multiplication as well as their fermentation at different temperatures. Some of these results are shown in table 1.

FERMENTATION

The amount of acid formed by large numbers of cells of *Lactobacillus* at 0° to 2° was within the limits of error. Since the plate counts showed only 6 million cells per cubic centimeter, this does not disprove their ability to ferment. All streptococci produced distinct amounts of acid at 0°[2] (figs. 1 and 2), but the fermentation ceased at a very low acidity. *S. fecalis*, *S. cremoris* and *S. liquefaciens* produced much more acid than the others. At 5°, 10° and 15°, the endpoints of fermentation became higher for each increase in temperature, but not proportionately, so that at 15°, *S. glycerinaceus* and *S. fecalis* were lowest, while at 0°, they were highest.

From these data and from the plate counts, the fermenting capacities (milligram lactic acid per cell per hour) were computed (table 2). The cells of the large inocula appear to have a greater fermenting capacity. This is partly due to the impossibility of redistributing centrifuged streptococci uniformly in milk; the plate counts indicate the number of clumps rather than individual cells. This accounts also for some irregularities which do not occur in the experiments with small inocula. It must further be remembered that old bacteria (in large inocula) lose their viability before they lose their fermenting power; this would make the fermentation per viable cell appear larger (Rahn and Barnes, 1933). Still another factor might cause differences: the bacteria for the experiments with large inocula had been grown at 30°, and it has never been proved that they really contain the same amount of fermenting zymases per cell as those grown at 5° or 10°.

Table 2 shows also the temperature coefficients of fermentation. By "temperature coefficient" is understood the increase

[2] This range will be abbreviated here as 0° which was the predominant temperature.

TABLE 1

Growth and acid formation of lactic bacteria at different temperatures (cells per cubic centimeter, acid in per cent of lactic acid)

	TIME	STREPTOCOCCUS FECALIS		STREPTOCOCCUS LACTIS		LACTOBACILLUS ACIDOPHILUS	
		Plate count	Per cent acid	Plate count	Per cent acid	Plate count	Per cent acid
°C.	days						
0	0	4,800	0	870	0		
	5	4,500	0	710	0		
	10	6,500	0				
	22	59,000	0	775	0		
	30	670,000	0	465	0		
	34	8,000,000	0				
	42	36,000,000	0.018	565	0		
	65	121,000,000	0.018	410	0		
	91	320,000,000	0.252	140	0		
	106	188,000,000	0.279	139	0		
5	0	4,800	0	900	0	70,000	0
	4	10,200	0	900	0	70,000	0
	6	31,600	0	2,000	0		
	9	187,000	0	3,900	0		
	12	1,138,000	0	13,000	0		
	14	4,900,000	0	28,000	0	86,000	0
	16	11,000,000	0	102,000	0		
	18	46,000,000	0	220,000	0		
	20	81,000,000	0	340,000	0	265,000	0
	22	143,000,000	0.036	510,000	0		
	24	270,000,000	0.090	1,000,000	0		
	33	305,000,000	0.126	10,000,000	0.018	240,000	0
	39	610,000,000	0.252			192,000	0
	42	345,000,000	0.270		0.036	115,000	0
	80		0.396			15,000	0
10	hours						
	0	4,800	0	880	0	70,000	0
	36	30,000	0	10,000	0		
	65	525,000	0	795,000	0		
	96	10,400,000	0	44,000,000	0.018		
	110	34,000,000	0.027	209,000,000	0.027		
	134	280,000,000	0.054			310,000	0
	182	790,000,000	0.126	425,000,000	0.090		
	220	890,000,000	0.180	360,000,000	0.108		
	342	870,000,000	0.342			7,900,000	0
	620		0.450			124,000,000	0.090
	955					191,000,000	0.252
	1477					144,000,000	0.558

TABLE 1—*Concluded*

	TIME	STREPTOCOCCUS FECALIS		STREPTOCOCCUS LACTIS		LACTOBACILLUS ACIDOPHILUS	
		Plate count	Per cent acid	Plate count	Per cent acid	Plate count	Per cent acid
°C.	hours						
15	0	4,800	0	880	0	70,000	0
	24			500,000	0		
	36			25,500,000	0		
	48			377,000,000	0.054		
	84	660,000,000	0.108	510,000,000	0.144		
	96	1,030,000,000	0.162	700,000,000	0.180		
	110	900,000,000	0.180	645,000,000	0.199		
	132	880,000,000	0.234	790,000,000	0.252		
	166	830,000,000	0.342	740,000,000	0.342	128,000,000	0
	200					170,000,000	0.054
	390		0.594			530,000,000	0.324
	529					770,000,000	0.558
	620					590,000,000	0.756
20	0	4,800	0	880	0	70,000	0
	16	4,500,000	0	4,500,000	0.018		
	25	120,000,000	0.018	289,000,000	0.036	380,000	0
	36	580,000,000	0.162	690,000,000	0.126		
	46	860,000,000	0.162	925,000,000	0.270	6,600,000	0
	54			1,117,000,000	0.324	14,500,000	0
	60	1,020,000,000	0.234	990,000,000	0.378	33,000,000	0
	65	1,120,000,000	0.270	890,000,000	0.414		
	100					193,000,000	0
	130	1,400,000,000	0.540			267,000,000	0.126
	200		0.630			450,000,000	0.450
	324					865,000,000	0.720
	464					530,000,000	0.936

in the rate of fermentation (or eventually of growth) for a temperature increase of 10°C. It is designated as Q_{10}. The temperature coefficient for 10° increase is the square of the temperature coefficient for 5° increase, i.e., $Q_{10} = (Q_5)^2$.

The most puzzling experience is the low endpoint of acidity in the cultures at 0°. Figure 2 shows the endpoints at different temperatures arranged in two groups with high and with low endpoint at 0°. It is clear that fermentation must be very slow. But since the enzymes are present in the cells, it is difficult to understand why fermentation should not go on with time and

reach ultimately as high an endpoint as it does at higher temperatures. All curves plainly indicate that this is not the case.

It seemed as if the enzymes in the cells were injured by prolonged cold. This was tested by holding milk cultures (inocu-

TABLE 2

Rates and temperature coefficients of lactic fermentation

	FERMENTING CAPACITIES (10^{-10} MGM. ACID PER CELL PER HOUR)							
	0°	5°	10°	15°	20°	30°	37°	40°
	Large inocula							
S. glycerinaceus	0.445	0.94	1.67	1.75				
S. liquefaciens	0.019	0.049	0.31	0.50				
S. fecalis	0.250	0.46	0.72	1.22				
S. lactis	0.200	0.61	1.67	3.44				
L. acidophilus	?	?	0.80	2.49	7.47			
	Small inocula							
S. fecalis	0.03	0.07	0.17	0.35	0.52	1.73	3.34	
S. lactis			0.38	1.05	2.78	8.10		
L. acidophilus			0.31	0.76	2.13	5.20	13.6	13.6
	TEMPERATURE COEFFICIENTS FOR 10° INCREASE							
	0° to 5°	5° to 10°	10° to 15°	15° to 20°	20° to 30°	30° to 37°	37° to 40°	
	Large inocula							
S. glycerinaceus	4.5	3.1	1.0					
S. liquefaciens	9.4	20.7	3.0					
S. fecalis	3.4	2.3	2.9					
S. lactis	9.0	7.5	4.2					
L. acidophilus			10.2	8.4				
	Small inocula							
S. fecalis	5.6	6.1	4.1	2.1	3.4	2.6		
S. lactis			7.7	7.0	2.9			
L. acidophilus			6.2	7.8	2.4	3.9	1.0	

lated with large numbers of cells as described above) for different lengths of time at 0°, and then raising the temperature suddenly to 30°C. and measuring the rate of fermentation. The experiment with *S. lactis* is given in detail in table 3. The acid of the 0° culture was neutralized just before raising the temperature.

The table shows that after one week, the bacteria were injured: the plate count decreased, and so did the fermenting capacity. In four weeks, the enzyme content per cell had dropped to one-fourth of the original. The six-week culture is for unknown reasons better preserved than any other.

At 30°, the injured cells replenish their enzyme content, but not completely as may be seen from the 2, 4 and 8 weeks old cultures compared with the first 6 hours of the 0 and 3 days old cultures. In the oldest culture, the enzyme content per cell still increases after 12 hours; the injury caused by the cold lasted through 5 generations of cells.

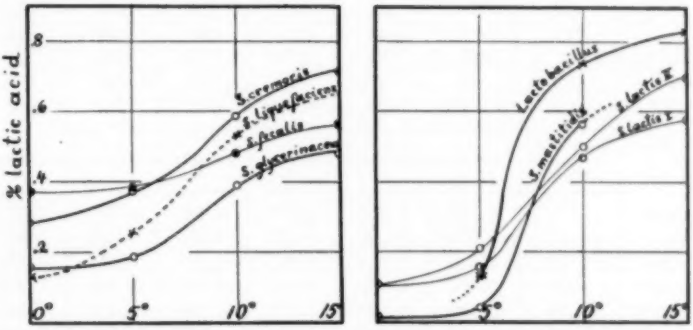


FIG. 2. MAXIMAL AMOUNTS OF ACID REACHED AT DIFFERENT TEMPERATURES BY INOCULATION WITH LARGE NUMBERS OF CELLS

Left: species producing high acidity at 0°. Right: species producing low acidity at 0°.

With *S. fecalis*, such injury could not be proved definitely. If it existed at all, it was slight. The difference between these two test organisms is that *S. fecalis* multiplies at 0°, and is therefore capable of repairing or replacing inactivated enzymes while *S. lactis* does not multiply at 0° (table 1).

GROWTH

As shown in table 1, only *S. fecalis* multiplies at 0° while *S. lactis* dies slowly. At 5°, *S. lactis* grew very slowly in 32 days to 10 million per cubic centimeter. The *Lactobacillus* multiplied

at 5° within 20 days from 70,000 to 265,000 cells per cubic centimeter, but no further, and after 36 days, the number gradually decreased. This probably corresponds to Orla-Jensen's statement (1919) that he observed growth of streptococci in glucose

TABLE 3

Effect of the time of holding at 0°C., on Streptococcus lactis

TIME OF HOLDING AT 0°	AFTER HOLDING	AFTER INCUBATION AT 30°C. FOR					
		2 hours	4 hours	6 hours	8 hours	12 hours	24 hours
		MILLIONS OF BACTERIA PER CUBIC CENTIMETER					
0 days	460	300	510	500	470	310	380
3 days	590	610	540	535	435	660	480
1 week	1,040	575	560	620	520	570	560
2 weeks	174	490	510	510	520	580	540
4 weeks	13.5	121	77	148	159	226	324
6 weeks	9.7	34	47	75	86	184	390
8 weeks	5.1	7.8	10	22	33	158	500
	(BEFORE NEUTRALIZING)	TOTAL, IN PER CENT OF LACTIC ACID, PRODUCED AFTER NEUTRALIZATION					
0 days		0.162	0.270	0.378	0.432	0.522	0.630
3 days	0.162	0.072	0.180	0.306	0.378	0.486	0.630
1 week	0.243	0.045	0.144	0.252	0.333	0.450	0.621
2 weeks	0.342	0.018	0.054	0.108	0.180	0.306	0.486
4 weeks	0.360	0.000	0.000	0.018	0.036	0.108	0.432
6 weeks	0.342	0.018	0.018	0.018	0.035	0.198	0.504
8 weeks	0.162	0.000	0.000	0.000	0.000	0.036	0.414

	FERMENTING CAPACITY IN 10^{-10} mgm.		
	0 to 6 hours	6 to 12 hours	12 to 24 hours
0 days	12.6	4.8	1.8
3 days	9.2	6.0	2.4
1 week	5.1	6.7	2.8
2 weeks	5.3	6.6	3.0
4 weeks	3.7	7.5	9.0
6 weeks	7.1	25.0	8.5
8 weeks	<1.7	8.7	10.6

broth without acid formation. It can be easily calculated from the fermenting capacity, 0.65×10^{-10} mgm., that the 265,000 cells per cubic centimeter observed would require 2700 days to produce 0.01 per cent lactic acid.

From the plate counts can be computed the maximal growth rates for each temperature, and from these the temperature coefficients of growth (table 4). The most rapid rate obtained is that by *S. lactis* at 30°; *S. fecalis* grows a little more slowly while the *Lactobacillus* requires about three times as many hours. Even at 10°, *S. fecalis* grows more slowly than *S. lactis* while at 5°, it grows twice as fast, and at 0°, it still grows while the other does not.

TABLE 4

Shortest generation times in hours

ORGANISM	0°	5°	10°	15°	20°	30°	37°	40°
S. fecalis	54.8	20.66	7.05	—	1.62	0.67	0.59	
S. lactis	∞	37.26	5.38	1.82	1.12	0.506		
L. acidophilus	∞	185.00	48.51	16.40	6.15	2.25	1.66	1.58
	TEMPERATURE COEFFICIENTS (Q_{10}) OF GROWTH							
	0° to 5°	5° to 10°	10° to 15°	15° to 20°	20° to 30°	30° to 37°	37° to 40°	
S. fecalis	7.0	8.6		4.4	2.4	1.2		
S. lactis	∞	48.2	8.7	2.6	2.2			
L. acidophilus	∞	14.5	8.7	7.1	2.7	1.5	1.2	

TABLE 5

Endpoints of growth, in millions of cells per cubic centimeter

	0°	5°	10°	15°	20°	30°	37°	40°
S. fecalis	320	610	900	1,030	1,120	1,095	1,130	
S. lactis		10	545	790	1,170	680		
L. acidophilus		0.28	170	770	795	1,230	1,200	940

The temperature coefficients show the usual picture of biological experiments; as the temperature approaches the minimum, Q_{10} increases distinctly above the value known for ordinary chemical reactions.

Interesting also is the effect of temperature on the "crop," i.e., the maximal number of cells (table 5). Near the minimum temperature, growth ceases early, and the crop is small. The largest crops were obtained over a range of about 10° from the temperature of fastest growth downwards.

DISCUSSION OF THE MINIMAL TEMPERATURE

A clear line must be drawn between the temperature responses of the growth processes and those of the processes furnishing energy. This is not always done. Bělehrádek (1935) gives an extensive compilation of minimum temperatures (biological zero) of biological processes arranged in groups where he distinguishes between "growth" and "metabolic processes," but places increase of chlorophyl and repair of injury under "metabolic processes." If these are placed under "growth," the minimum temperature of metabolic processes is almost always the freezing point, while that for growth is almost always higher. This should be expected. Enzymatic processes such as respiration or fermentation, though they may take place within the cell, should hardly be handicapped by hardships of the cell. Growth processes are so interlinked that the retardation of any single reaction might prevent completely the functioning of others.

However, the rate of fermentation in our experiments is *progressively* slower at low temperatures while the temperature coefficient of chemical reactions changes so little in the narrow range of biological events that it is constant for all practical purposes. It has been shown in table 3 that within a week at 0°, the enzyme content of the cell is decreased. The rates of fermentation have been computed, however, from the amount of acid formed during the first days, where injury can have been only slight. The temperature coefficients between 0 and 5° (table 2) are lowest with the two streptococci which grow at 0°, and which consequently can repair and replenish the enzyme while the two species which cannot do this show a high temperature coefficient. Still, this is not quite sufficient to prove that the rate of fermentation has really a constant temperature coefficient, and that only such events as the gradual deterioration interfere.

As to the lower final acidity at lower temperatures, the injury of the enzyme through cold is not quite sufficient for an explanation. It suffices only for those temperatures where cells do not multiply while even with *S. fecalis* (fig. 1), the final acidity increases from 0 to 15°. The common assumption regarding the endpoint of fermentation is that the concentration of products

prohibits further action of the enzyme. If decreased temperature had any effect at all, we should expect it to increase the endpoint. We are coping here with an unknown factor which may be due to a change of equilibrium in the enzyme reaction, or to a change in the water itself though water polymers are probably not the cause.

The reason for the cessation of growth at 5° by *S. lactis* and *L. acidophilus* while *S. fecalis* grows even at 0° is still more difficult to find. Since the fermentation below 5° is greatly decreased, it seemed possible that not enough calories were produced to supply the growth mechanism with the minimum energy required for synthesis. This led to the calculation of the amount of energy needed for the synthesis of one cell. Streptococci grow so very poorly without carbohydrates that it seems safe to consider the lactic fermentation as practically the only source of energy. If we multiply the generation time (in hours, as given in table 4) by the milligrams of lactic acid per cell per hour, we have the amount of acid formed, or of sugar consumed, for the formation of one cell. The results of this computation are given in table 6. It reveals the surprising fact that the bacteria do not require more food at low temperatures. At low or medium temperatures, the energy consumption is constant, but at temperatures near the optimum, the food consumption per generation increases; the bacteria utilize their food less economically.

TABLE 6

Lactose required for the doubling of one cell recorded in 10^{-10} mgm.

	0°	5°	10°	15°	20°	30°	37°	40°
S. fecalis	1.1	1.4	1.2	—	0.8	1.2	2.0	
S. lactis	—	—	2.0	1.9	3.1	4.1		
L. acidophilus	—	—	14.8	12.5	13.1	11.7	22.6	21.5

If this constant sugar requirement were extrapolated below 5° for *S. lactis*, we should still have growth at 0°, very slow to be sure, but not impossibly slow. The product of fermenting capacity × generation time is a constant, namely, 2×10^{-10} mgm. From 5° to 0°, the fermenting capacity drops to one-

third, consequently the generation should increase 3 times, which would make it 112 hours. Such generation times at low temperatures are not impossible. Therefore, since this event does not take place, it is not the fault of too slow fermentation.

S. fecalis utilizes the lactose most economically. *S. lactis* needs almost twice as much lactose, and the large *Lactobacillus* 10 times as much in order to make two cells out of one.

The most common explanation of growth cessation due to low temperatures is probably the assumption that the various interlinking reactions are influenced differently by a change of temperature, and that they may get "out of step" and upset the growth mechanism. Aside from this Bělehrádek lists four other explanations:

(1) Accumulation of toxic products in the cell (this might be the result of the disturbed growth mechanism just mentioned).

(2) Change in permeability or ion absorption.

(3) Solidification of important lipids.

(4) Too great viscosity of protoplasm.

This last point, he states as follows: "Such an increase (in viscosity) would considerably hinder free movements of reacting molecules with the result that the biochemical reactions in the cell would be brought to a standstill."

This statement is far too general. "Free movement" will usually be interpreted as meaning the rate of diffusion. As far as crystalloids are concerned, this is not hindered appreciably by an increase in viscosity, not even by a change from sol to gel (Gortner, 1929, p. 210). If the term "free movement" is to include selective permeability of the membranes, we are facing a different problem which is probably linked with point (3), solidification of lipids.

Point (1) could hardly be considered with organisms having such an enormous surface as bacteria; any product of normal or abnormal cell metabolism could easily diffuse out. Points (2) and (3) can probably not be determined with such small organisms as bacteria.

SUMMARY

When large numbers of cells of streptococci are placed in milk at low temperature, lactic acid fermentation takes place even at 0° with all species tested.

The rate of fermentation is greatly decreased at 0°, more so with the species which cannot grow at 0° than with those that do.

With *Streptococcus lactis*, incapable of growing at 0°, the enzyme content is distinctly decreased by one week's holding at 0°, and after 4 to 8 weeks, the cells require a number of generations before they come back to their original fermenting capacity. With *Streptococcus fecalis*, capable of multiplying at 0°, such an effect was not noticed.

The total acid produced by each species is lowest at 0°, and increases with temperature. The difference is smaller with those species which grow at 0°, but it is noticeable in all cases (fig. 2) and cannot be explained merely by a deterioration of the enzyme.

No explanation can be given for the fact that *Streptococcus fecalis* and *Streptococcus glycerinaceus* grow at 0° while *Streptococcus lactis* and *Lactobacillus acidophilus* die at this temperature, and can hardly multiply even at 5°.

The amount of lactose consumed during the doubling of one cell is constant at low and medium temperatures, but increases towards the optimal temperature. *Streptococcus lactis* requires twice as much lactose as *Streptococcus fecalis*, and *Lactobacillus acidophilus* needs 10 times as much to double its cell.

The largest bacterial crops were obtained at temperatures between the optimum and 10° below the optimum. Near the minimum, the crops decreased decidedly.

REFERENCES

BĚLEHRÁDEK, J. 1935 Temperature and Living Matter. Berlin, Borntraeger.
GORTNER, R. A. 1929 Outlines of Biochemistry. New York, Wiley and Sons.
ORLA-JENSEN, S. 1919 Lactic Acid Bacteria. Copenhagen, Host and Son.
RAHN, OTTO 1932 Physiology of Bacteria. Philadelphia, Blakiston.
RAHN, OTTO, AND BARNES, M. N. 1933 Jour. General Physiol., **16**, 579.

SUGAR ALCOHOLS. VI

THE UTILIZATION OF SUGAR ALCOHOLS AND THEIR ANHYDRIDES BY VARIOUS MICROÖRGANISMS

K. PIERRE DOZOIS, C. JELLEFF CARR, JOHN C. KRANTZ, JR., FRANK HACHTEL AND FRANCES F. BECK

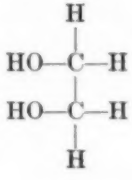
Departments of Bacteriology and Pharmacology, School of Medicine, University of Maryland

Received for publication June 5, 1936

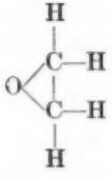
In a former communication the authors (1935) presented studies on acid and gas formation by members of the colon-aerogenes-intermediate groups in the presence of sugar alcohols and their anhydrides. In general it was observed that mannitol and dulcitol are fermented by many organisms of this group, but that their anhydrides are not utilized as determined by ordinary fermentation tests. So striking was the resistance to utilization of the anhydrides in contrast to the ease of metabolism of the sugar alcohols themselves that the study was extended in order further to elucidate the relationship between chemical constitution and fate in the bacterial economy.

CHEMICAL CONSTITUTION

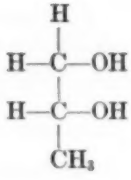
The following formulas show the structural relationships of the substances employed.



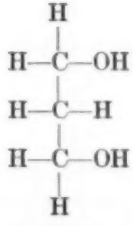
ethylene glycol



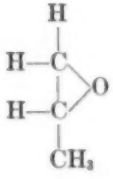
ethylene oxide



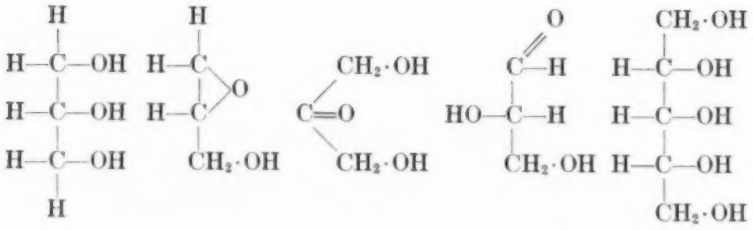
propylene glycol



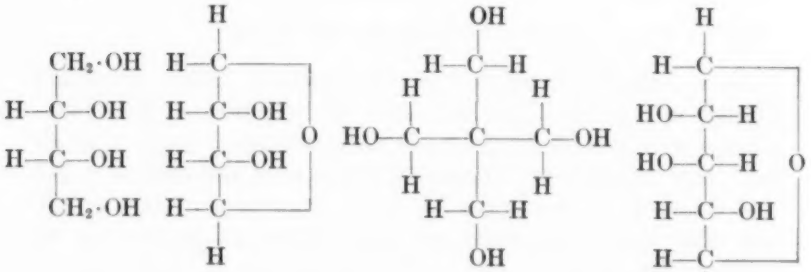
trimethylene glycol



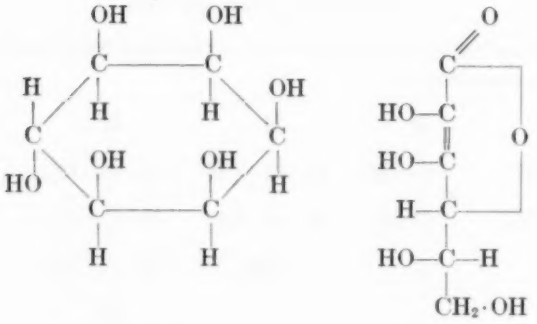
propylene oxide



glycerol | l-epihydrin alcohol | dihydroxy-acetone | d,l-glyceric aldehyde | i-adonitol



i-erythritol | erythritan | pentaerythritol | polygalitol



i-inositol | l-ascorbic acid

The inositol, erythritol, pentaerythritol and adonitol employed were Pfanstiehl's C.P. products and met the standard specifications of their catalogue. Ethylene glycol, ethylene oxide, propylene glycol, propylene oxide and trimethylene glycol were supplied by the Eastman Kodak Company. The dihydroxyacetone employed was "oxantin" prepared formerly by Merck and Company. The epihydrin alcohol was prepared from epibromhydrin (1915), and its l-form distilling at 70° at 10 mm. pressure was used. The polygalitol was extracted from *Polygala amara* (1927). The compound melted at 138° uncorrected. The erythritan was prepared from erythritol by dehydration with sulfuric acid (1936). Analysis gave the following composition: C 45.21 per cent; H 7.65 per cent. The calculated values are C 46.15; H 7.69. The l-ascorbic acid was supplied through the courtesy of Joseph Rosin, Merck and Company, Rahway, New Jersey. It is marketed under the name of cevitamic acid. The glyceric aldehyde, prepared by Drs. Frankel and Landau, was supplied to us through the kindness of Dr. E. K. Marshall, Jr., of Johns Hopkins Medical School.

METHOD OF STUDY

As in our previous studies, 1-per-cent carbohydrate broth culture media were prepared by adding the sterile carbohydrates to sugar-free nutrient broth. The cultures, after inoculation, were incubated for ten days and observations for the formation of acid and gas were made daily. With the ascorbic acid media, acid formation could not be readily measured and only observations of gas formation and growth were made. Thirty strains of the colon-aerogenes-intermediates, three of *Eberthella typhosa*, and four of *Salmonella* were studied. These were distributed among the species as indicated in table 1.

RESULTS

Of the species studied, none was able to ferment ethylene oxide, propylene oxide, trimethylene glycol, epihydrin alcohol, erythritol, erythritan or pentaerythritol. All species of *Aerobacter* and A and D strains of the intermediate group formed acid with

ethylene glycol. Dihydroxyacetone was fermented with the production of acid and gas by *Escherichia* and *Aerobacter* and by strains A and D of the "intermediates." Only the cultures of *Escherichia* and *Aerobacter* were able to form acid from propylene

TABLE 1

ORGANISMS STUDIED	ETHYLENE OXIDE	ETHYLENE GLYCOL	DIHYDROXY ACETONE	GLYCERIC ALDEHYDE	PROPYLENE OXIDE	PROPYLENE GLYCOL	TRIMETHYLENE GLYCOL	GLYCEROL	EPIHYDRIN ALCOHOL	ERYTHRITOL	ERYTHRITAN	PENTAERYTHRITOL	ADONITOL	POLYGALITOL	INOSITOL	ASCORBIC ACID
Escherichia																
coli	–	–	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
communior	–	–	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
neapolitana	–	–	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
enterica	–	–	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
alba	–	–	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
Aerobacter																
aerogenes	–	A	A+	A	–	A	–	A+	–	–	–	–	A+	A+	A+	G
cloacae	–	A	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
levans	–	A	A+	A	–	A	–	A+	–	–	–	–	–	A+	A+	G
oxytocum	–	A	A+	A	–	A	–	A+	–	–	–	–	A+	A+	A+	G
liquefaciens	–	A	A+	A	–	A	–	A	–	–	–	–	–	A+	A+	G
Intermediate																
A	–	A	A+	A	–	–	–	A+	–	–	–	–	A+	A	A+	G
B	–	–	A	A	–	–	–	A+	–	–	–	–	–	A	–	G
C	–	–	A	A	–	–	–	A+	–	–	–	–	–	A+	–	G
D	–	A	A+	A	–	–	–	A+	–	–	–	–	A+	A+	A+	G
Eberthella																
typhosa A	–	–	A	A	–	–	–	–	–	–	–	–	–	A	–	G
typhosa B	–	–	A	A	–	–	–	–	–	–	–	–	–	A	–	G
typhosa C	–	–	A	A	–	–	–	–	–	–	–	–	–	A	–	G
Salmonella																
paratyphi	–	–	A	A	–	–	–	A+	–	–	–	–	–	A+	–	G
Schottmuelleri	–	–	A	A	–	–	–	A+	–	–	–	–	–	A+	A+	G

– = no fermentation; A = acid formation; A+ = acid and gas formation; G = growth.

glycol, and all the organisms formed acid from glyceric aldehyde. With the exception of *Eberthella typhosa* which was inactive on glycerol and *Aerobacter liquefaciens* which produced only acid, all the other organisms formed acid and gas from glycerol. Strains A and B of the intermediate group and *Eberthella typhosa* formed

acid from polygalitol, while the other cultures produced both acid and gas. *Eberthella typhosa*, *Salmonella paratyphi* and "intermediates" B and C failed to ferment inositol. *Aerobacter aerogenes*, *Aerobacter oxytocum*, and A and D strains of "intermediates" fermented adonitol. Although ascorbic acid did not inhibit growth, none of the microörganisms was capable of producing gas from it.

The action of the *Escherichia-Aerobacter*-intermediate groups on propylene glycol is of special interest as it may lead to a further means of identification of the "intermediates." The uniform action of strains A and D of the "intermediates" on ethylene glycol, dihydroxyacetone, adonitol and inositol leads one to believe that they are distinct strains and may be differentiated from B and C on the basis of this action.

A study of these results compared with our previous findings reveals the interesting fact that although neither acid nor gas was formed from the anhydrides of mannitol, i.e., mannitan, mannide, isomannide, both acid and gas were produced from the 1–5 anhydride, polygalitol. Mannitan, mannide and isomannide possess a distinctly bitter taste; polygalitol on the other hand is sweet like the parent substance, mannitol.

CONCLUSIONS

1. In general, our previous findings have been extended and confirmed, namely that the removal of a molecule of water from sugar alcohols with the formation of the oxide ring prevents their utilization by many organisms of the colon-aerogenes group.

2. Polygalitol (1–5 anhydromannitol) stands as an exception to this rule.

3. The inability of the "intermediates" to ferment propylene glycol may lead to a method of distinguishing them from the *Escherichia* and *Aerobacter*.

REFERENCES

ABDERHALDEN AND EICHWALD 1915 Ber., **48**, 1847.

BECK, F. F. 1936 A Thesis. University of Maryland. "The fate of erythritol and erythritan in the animal body."

DOZOIS, K. P., HACHTEL, FRANK, CARR, C. J., AND KRANTZ, J. C., JR. 1935 Jour. Bact., **30**, 189.

PICARD 1927 Bull. Soc. Chim. Biol., **9**, 692.

THE ABSORPTION OF STAPHYLOCOCCUS BACTERIOPHAGES[1]

MORRIS L. RAKIETEN, TONY L. RAKIETEN AND SIMON DOFF

Department of Immunology, Yale University School of Medicine and Department of Bacteriology, Long Island College of Medicine, Brooklyn, New York

In a series of papers beginning in 1933 Levine and Frisch have affirmed that extracts derived from susceptible cultures of *Salmonella* block the lytic activity of bacteriophages active against that group of bacteria. These investigations have been confirmed by Burnet (1934), utilizing organisms and phages belonging to the same group as well as several strains of *Shigella*. These extracts apparently reflect quite accurately those surface antigenic components responsible for bacteriophage absorption on living and heat-killed susceptible cultures.

In the course of our studies with staphylococcus bacteriophages we have been struck by the fact that these phages are exceedingly difficult to isolate from sources rich in phages for members of the enteric group. It may very well be that one of the factors contributing to this difficulty is the absorbing quality of such substances as molds, yeasts, phage-resistant staphylococci, and body exudates (Applebaum and MacNeal, 1931; Rakieten, 1932). Other bacteriophages, especially those attacking organisms of the enteric group are not affected at all by the same agents that have a capacity for what may be termed "non-specific absorption" of staphylococcus phages.

The absorption of staphylococcus phages by organisms other than staphylococci (to be reported later) has revealed evidence of heterogenetic antigenic relations between staphylococci and otherwise totally unrelated organisms. This report deals with (1) the absorption and inactivation of staphylococcus bacteriophages by extracts prepared from susceptible and resistant strains

[1] Presented in part before the Society of American Bacteriologists, New York, December, 1935.

of staphylococci, and (2) the absorption of similar phages by heat-killed susceptible and resistant cultures belonging to the same group of organisms.

1. STUDIES WITH BACTERIAL EXTRACTS

The early reports of Levine and Frisch concerning the preparation of bacterial extracts resulted in our attempt to apply their technique to phage-susceptible strains of staphylococci. All such attempts resulted in preparations weak in phage-inhibiting power, or lacking this property. The method of Burnet (1934) yielded extracts whose phage-inactivating power closely paralleled the susceptibility of the culture to bacteriophagy.

TECHNIQUE

Bacteria were grown on 1.5 per cent Savita agar[2] (Rakieten, 1932) in Roux bottles (150 to 200 cc. per bottle) for 48 hours at 35°C. and then washed off with 10 cc. of sterile distilled water. The washings were placed in a 56° to 57°C. water-bath for 48 hours. Bacteria which settled rapidly were resuspended by occasional shaking. After 48 hours the autolysates were centrifuged at 3000 r.p.m. for one hour and the clear to slightly opalescent supernatant fluid was removed. Only those extracts which on testing proved to be free of living bacteria were used.

For demonstrating the effect of the extracts on several races of bacteriophage serial dilutions of the extracts were prepared, broth being used as the diluent, and to each dilution of extract an equal volume of bacteriophage was added. The bacteriophage was so diluted that at least 200 to 500 plaques could be counted in the final mixture, provided no inhibitory effect had occurred. After the extract-bacteriophage mixtures were incubated for 20 hours at 35°C., 0.02 cc. of each mixture was layered over a segment of an agar plate previously seeded with a susceptible culture in such a manner that a homogeneous layer was provided for the demonstration of plaques. After a further incubation for 20 hours at 35°C. the plates were counted for plaques.

A method which reveals its effectiveness by a diminution in

[2] Unless otherwise noted media used in this study were Savita broth and agar.

plaque count is certainly a better criterion of inactivation than are standards of turbidity in a test-tube. In appearance, secondary cultures may often correspond to controls uninoculated with bacteriophage, and yet contain practically as much phage by plaque count as does a tube showing complete lysis. Such tubes would be read as absence of bacteriophagy. It has been demonstrated by d'Herelle (1920), Eliava and Pozerski (1921), Asheshov (1933), and others, that it is possible to isolate from secondary cultures organisms which are completely susceptible. Only when the entire secondary culture is used is resistance to the bacteriophage, responsible for the appearance of the secondary culture, demonstrable consistently.

Experimental

A. To equal quantities of increasing dilutions of extracts of 8 strains of susceptible staphylococci, an equal and constant amount of bacteriophage was added, the final volume being 2 cc. After incubation for 20 hours at 35°C., 0.02 cc. from each tube of each series was layered over homologous cultures previously streaked on segments of agar plates. Plaque counts were made after 20 hours at 35°C. The results are summarized in table 1.

These results corroborate those of Levine and Frisch and of Burnet: extracts of susceptible cultures possess phage-blocking power. The most potent extracts were derived from those strains which, on the whole, were the most susceptible. In this category we would place Staphylococcus H43, H741, Vnt., and PTR, since they are the ones that are lysed in the shortest period and do not give rise to secondary cultures. The remaining cultures (with the exception of Staph. v), although susceptible, give rise in most instances to secondary cultures after a period of 48 hours. Their phage-inactivating power, or rather the potency of their extracts in this direction is considerably less than is that of the other strains. Staph. v, the only *Staphylococcus albus* in this series is completely susceptible and never gives rise to secondary culture, even on prolonged incubation. It constitutes an exception in that its extract, which on the basis of strain susceptibility ought to be as powerful in blocking phage M15 as some of the other strains, is relatively weak.

B. All of the extracts in this series were obtained from cultures susceptible to our polyvalent phages, but with the exception of Staphylococcus v, these cultures are resistant to Bacteriophage V. Extract v is derived from strain v, which is susceptible to phage V and to all of our other staphylococcus phages. Hence, with the exception noted above, all of these extracts may be considered to have been derived from cultures resistant to Bacteriophage V.

The technique used is that described above, except that the extract-phage mixtures were plated on Staph. v, it being the only

TABLE 1

Effect of extracts on bacteriophage M15

EXTRACT (FINAL DILUTION)	NUMBER OF PLAQUES IN 0.02 CC. OF EXTRACT-PHAGE MIXTURE, HOMOLOGOUS SUSCEPTIBLE CULTURES							
	Staph. v	Staph. Pg.	Staph. Vnt.	Staph. H43	Staph. H741	Staph. Arc.	Staph. Sch.	Staph. PTR.
1:2	28	2	0	1	0	6	1	0
1:5	108	6	0	2	0	12	7	0
1:10	20	15	0	2	0	14	52	0
1:20	347	85	0	4	0	10	206	10
1:40	411	253	0	0	0	27	254	31
1:80	557	401	1	4	0	120	300	136
1:160	NT	457	42	16	10	259	357	184
1:320	NT	NT	256	111	105	418	403	400
1:640	NT	NT	277	207	197	NT	NT	NT
1:1280	NT	NT	304	NT	NT	NT	NT	NT
Bacteriophage control	500	401	301	255	241	490	500	500

NT (not tested in this dilution).

culture susceptible to Bacteriophage V. The results are indicated in table 2.

On the basis of these results one may conclude that extracts derived from strains of staphylococci not susceptible to a phage lack on the whole, significant phage-inactivating power. The one susceptible culture, v, does provide an extract that in dilutions as high as 1:20 markedly reduces corpuscular count. Test-tube methods show that this extract, in a dilution of 1:20, plus phage V and the susceptible culture prevents lysis. In higher dilutions of extract, under the same conditions, lysis in the test-tube takes

place. While a slight reduction in plaque count is noted in high concentrations of extracts of some of the non-susceptible cultures, test-tube experiments fail to show any inhibitory action of these extracts on phage V. The diminution may be due to a slight amount of susceptible substance in these cultures, not demonstrable except by experiments involving extracts.

C. Extracts have been prepared from three completely resistant staphylococci, i.e., strains resistant to all of our races of polyvalent phages. As far as could be determined, extracts prepared from these three strains failed to inhibit the action of any of these phages when they were subsequently tested either for diminution of plaque count, or lysis in the test-tube.

TABLE 2

Effect of staphylococcus extracts on monovalent phage V

EXTRACT (FINAL DILUTION)	NUMBER OF PLAQUES IN 0.02 CC. OF EXTRACT-PHAGE MIXTURE SUSCEPTIBLE CULTURE V				
	Staph. Vnt.	Staph. Pg.	Staph. Sch.	Staph. v	Staph. H741
1:2	157	144	211	4	137
1:10	157	139	181	60	194
1:20	195	111	207	76	217
1:40	217	177	NT	144	NT
1:80	NT	241	NT	201	NT
1:160				227	
1:320				222	
Bacteriophage V control				236	

D. The results recorded have been obtained many times with these and with 12 other extracts prepared from susceptible cultures of staphylococci. Extracts from susceptible cultures always successfully inactivated bacteriophage, whereas extracts from resistant cultures, with one exception, lacked this property. This unusual culture is a *Staphylococcus albus*, which is not only completely resistant to all of our races of phages, but within a period of 3 to 8 hours is able to absorb completely the phage without showing lysis of any degree. Filtrates of this culture to which phage has been added and incubated for 8 hours are devoid of lytic action, whereas phage control filtrates contain millions of

B. All of the extracts in this series were obtained from cultures susceptible to our polyvalent phages, but with the exception of Staphylococcus v, these cultures are resistant to Bacteriophage V. Extract v is derived from strain v, which is susceptible to phage V and to all of our other staphylococcus phages. Hence, with the exception noted above, all of these extracts may be considered to have been derived from cultures resistant to Bacteriophage V.

The technique used is that described above, except that the extract-phage mixtures were plated on Staph. v, it being the only

TABLE 1

Effect of extracts on bacteriophage M15

EXTRACT (FINAL DILUTION)	NUMBER OF PLAQUES IN 0.02 CC. OF EXTRACT-PHAGE MIXTURE, HOMOLOGOUS SUSCEPTIBLE CULTURES							
	Staph. v	Staph. Pg.	Staph. Vnt.	Staph. H43	Staph. H741	Staph. Arc.	Staph. Sch.	Staph. PTR.
1:2	28	2	0	1	0	6	1	0
1:5	108	6	0	2	0	12	7	0
1:10	20	15	0	2	0	14	52	0
1:20	347	85	0	4	0	10	206	10
1:40	411	253	0	0	0	27	254	31
1:80	557	401	1	4	0	120	300	136
1:160	NT	457	42	16	10	259	357	184
1:320	NT	NT	256	111	105	418	403	400
1:640	NT	NT	277	207	197	NT	NT	NT
1:1280	NT	NT	304	NT	NT	NT	NT	NT
Bacteriophage control	500	401	301	255	241	490	500	500

NT (not tested in this dilution).

culture susceptible to Bacteriophage V. The results are indicated in table 2.

On the basis of these results one may conclude that extracts derived from strains of staphylococci not susceptible to a phage lack on the whole, significant phage-inactivating power. The one susceptible culture, v, does provide an extract that in dilutions as high as 1:20 markedly reduces corpuscular count. Test-tube methods show that this extract, in a dilution of 1:20, plus phage V and the susceptible culture prevents lysis. In higher dilutions of extract, under the same conditions, lysis in the test-tube takes

place. While a slight reduction in plaque count is noted in high concentrations of extracts of some of the non-susceptible cultures, test-tube experiments fail to show any inhibitory action of these extracts on phage V. The diminution may be due to a slight amount of susceptible substance in these cultures, not demonstrable except by experiments involving extracts.

C. Extracts have been prepared from three completely resistant staphylococci, i.e., strains resistant to all of our races of polyvalent phages. As far as could be determined, extracts prepared from these three strains failed to inhibit the action of any of these phages when they were subsequently tested either for diminution of plaque count, or lysis in the test-tube.

TABLE 2

Effect of staphylococcus extracts on monovalent phage V

EXTRACT (FINAL DILUTION)	NUMBER OF PLAQUES IN 0.02 CC. OF EXTRACT-PHAGE MIXTURE. SUSCEPTIBLE CULTURE V				
	Staph. Vnt.	Staph. Pg.	Staph. Sch.	Staph. v	Staph. H741
1:2	157	144	211	4	137
1:10	157	139	181	60	194
1:20	195	111	207	76	217
1:40	217	177	NT	144	NT
1:80	NT	241	NT	201	NT
1:160				227	
1:320				222	
Bacteriophage V control				236	

D. The results recorded have been obtained many times with these and with 12 other extracts prepared from susceptible cultures of staphylococci. Extracts from susceptible cultures always successfully inactivated bacteriophage, whereas extracts from resistant cultures, with one exception, lacked this property. This unusual culture is a *Staphylococcus albus*, which is not only completely resistant to all of our races of phages, but within a period of 3 to 8 hours is able to absorb completely the phage without showing lysis of any degree. Filtrates of this culture to which phage has been added and incubated for 8 hours are devoid of lytic action, whereas phage control filtrates contain millions of

corpuscles. An 18-hour culture heated at either 60°C. for an hour, or autoclaved at 15 pounds pressure for 15 minutes will absorb phage completely in 24 hours at 35°C. Extracts were prepared from this culture several times in 1935 and again recently; none has shown phage-inactivating power. Levine and Frisch (1933) have noted that in one case, at least, an extract from a phage-resistant strain possessed inactivating properties. Burnet (1934) also encountered the same sort of an exceptional strain, an R variant of a Y strain of *Shigella paradysenteriae*.

According to Burnet the phage-inactivating principle of these extracts is precipitated by the addition of the homologous antibacterial serum, and our observations are in accord with this statement, the supernatant fluids in no way blocking the lytic action of bacteriophage. A 1:20 dilution of rabbit anti-staphylococcus v serum was diluted with an equal volume of staphylococcus extract v. The mixture and the controls were held in a 55°C. bath for an hour and in the refrigerator overnight, whereupon the finely suspended aggregates were thrown down and the supernatant fluids removed. Equal quantities of staphylococcus phage V were added to the supernatants, incubated for 20 hours at 35°C. and finally 0.02 cc. of each was layered over a segment of an agar plate previously treated with susceptible culture v. After incubation at 35°C. for 18 hours plaque counts were made. The results are recorded in table 3.

TABLE 3

Removal of phage-inactivating power of extract v by anti-serum

	PLAQUE COUNT ON STAPH. V
Serum 255 (1:40) plus extract v (1:2); plus Staph. Bp. V.....	249
Extract v plus Staph. Bp. V.....	1
Control Bp. V.....	217

Similar results may be obtained with other extracts and other sera providing the latter contain agglutinins and precipitins for the organisms providing the extracts.

Once the bacteriophage has been inactivated by an extract it

has not been possible by the dilution method to show any increase in corpuscular count. In this respect inactivation by extracts differs from neutralization by anti-phagic serum (Andrews and Elford, 1933).

All of the extracts are deprived of phage-inactivating ability by filtration through Chamberland L3, and L5 candles, Berkefeld V, and Seitz filters, but potency is not diminished by heating for an hour at temperatures from 60 to 100°C. Activity is only slightly lessened when the extract is autoclaved at 15 pounds for 15 minutes.

2. ABSORPTION OF STAPHYLOCOCCUS BACTERIOPHAGES BY HEAT-KILLED CULTURES OF STAPHYLOCOCCI

It has been demonstrated that heat-killed susceptible cultures will absorb bacteriophage, (Da Costa Cruz, 1922; Jaumain and Meuleman, 1922; Bail and Okuda, 1923; Prausnitz and Firle, 1924; Burnet, 1930; Levine and Frisch, 1934). It is also agreed that with few exceptions lyso-resistant strains (secondary cultures) do not bind homologous phage (d'Herelle, 1921; Jaumain and Meuleman, 1922). Our interest in this problem aside from showing that staphylococcus phages behave as do those active with other bacterial species, centered on the question as to whether bacteriophage absorbed to dead cells may under suitable conditions be liberated and proliferate at the expense of living susceptible bacteria. If this could be demonstrated experimentally one might conclude that dead bacteria may serve as carriers of phage and that outside of the test-tube this union might, under certain conditions, be broken with the subsequent liberation of the active lytic agent.

Experimental evidence regarding the absorption of staphylococcus phage by heat-killed susceptible cultures is easily obtainable especially if the tests are made at 35°C. It appears that the most susceptible of our strains, when killed at 60°C. may even absorb phage at ice-box temperature. Strains that are only partially susceptible under no circumstances completely absorb phage. The absorption is not entirely irreversible, for active corpuscles may be demonstrated if susceptible living cells are

added to the tubes containing heat-killed culture-absorbed phage mixtures. When the absorption is carried out at 20°C. no plaques are demonstrable; yet complete lysis of susceptible cultures may occur when they are added, and with this there is a multiplication of phage corpuscles.

EXPERIMENTAL

A. Absorption of phage M16 by heat-killed cultures. Nine strains of staphylococci, which, with one exception (strain Gre.), are completely lysable by bacteriophage M16 were tested for their ability to absorb bacteriophage after being heated at 60°,

TABLE 4

Absorption of phage M16 by heat-killed (62°C. for an hour) staphylococci

CULTURE	INCUBATED 35°C., 22 HOURS, PLAQUE COUNT	ICE BOX (4°C., 22 HOURS), PLAQUE COUNT
Staphylococcus aureus Sch	0	0
Staphylococcus aureus Arc	1	101
Staphylococcus aureus PTR	0	7
Staphylococcus aureus Mtg	0	0
Staphylococcus aureus Pg	0	0
Staphylococcus aureus H741	0	0
Staphylococcus aureus H43	3	145
Staphylococcus albus v	1	62
Staphylococcus albus Gre	20	500
Control Bp. (Staph. aur. Vnt. used as susceptible strain)	500	500

80° and 100°C. for an hour, and after being autoclaved at 15 pounds for 15 minutes. Eighteen-hour broth cultures, after being heated at 62°C. for an hour were centrifuged, the supernatant fluids removed, and the sedimented bacteria resuspended in 5 cc. of fresh broth. The supernatants were devoid of phage-binding power. To 5 cc. of broth, 2 cc. of the bacterial suspension and 1 cc. of bacteriophage were added, the bacteriophage being so diluted that 0.02 cc. would yield approximately 500 plaques providing no absorption had taken place. The mixtures were placed at 35°C. and in the ice box for 22 hours, when 0.02 cc. was layered over a segment of an agar plate previously seeded with susceptible

culture. The results of such an experiment are indicated in table 4.

It is clear that susceptible cultures of staphylococci heated for one hour at 62°C. are capable of absorbing phage, especially when tests are made at incubator temperatures, although there is also a very marked degree of absorption to all of the totally susceptible cultures even at temperatures as low as 4°C. *Staphylococcus* Gre., an albus variety, is but incompletely lysed by phage M16, and shows no bacteriophage-binding power at ice-box temperature. Similar results were obtained with the susceptible cultures when they had been autoclaved and were tested for absorption. These results with our autoclaved cultures are not in accordance with those of Prausnitz and Firle (1924) who reported that phage-susceptible cultures lose their ability to absorb phage when heated to 100°C. or above. It is interesting to recall at this point that extracts made from some of the above strains also retained their phage-inactivating power, when autoclaved for the same period, and also to note, as described in a preceding section, that a living culture of *Staphylococcus albus* (Gec.) may absorb phage without undergoing lysis. This strain when autoclaved also absorbs phage, but an extract of this culture has no inactivating power.

Having demonstrated that heat-killed susceptible cultures absorb phage, attempts were made to ascertain whether such absorbed phage could be freed and bring about bacteriophagy, as evidenced by increase in plaque count, or lysis in the test-tube.

All experiments to free phage from heat-killed susceptible culture, by such means as plasmolysis, or plasmoptysis failed. However, it is interesting to note that bacteriophage controls were undiminished in titer for as long as 11 days in distilled water, or hypertonic or hypotonic salt solution.

The presence of free lytic principle could be demonstrated by the addition of a small amount of living susceptible culture to the tubes containing the absorbed phage. This resulted not only in the appearance of plaques but also in some instances in complete lysis in the test-tube.

Experiment. An 18-hour broth culture (10 cc.) of Staph. Vnt. was killed at 62°C. for one hour. After centrifugation the super-

natant was removed and the bacteria resuspended in one-half the original volume of fresh broth. Of this suspension, 0.25 cc. plus an equal volume of bacteriophage were added to 5 cc. of broth and incubated for 20 hours at 35°C. A test for absorption was then made by overlaying 0.02 cc. on a susceptible culture. To the absorbed phage-culture mixture was added 0.05 cc. of an 18-hour culture of Staph. Vnt. (susceptible). The tubes with controls were then incubated for 20 hours at 35°C., being observed at frequent intervals for macroscopic evidence of lysis. They were then filtered through L5 Chamberland candles and the fil-

TABLE 5

Test	*Control 1*
Staph. Vnt. (heat-killed) 0.25 cc. plus 0.25 cc. of Bp. M16. Incubated 35°C., 20 hours. Plaque test reveals no plaques. 0.05 cc. of Staph. Vnt. added, incubated 35°C., 20 hours. Test-tube shows no evidence of lysis. Filtrate (0.02 cc.) overlayed on Staph. Vnt. shows after incubation 400 plaques.	Bp. M16, 0.25 cc. incubated 35°C., 20 hours. Plaque test reveals in 0.02 cc. approximately 400 plaques. Staph. Vnt. added, incubated 35°C., 20 hours. Test-tube shows complete lysis. 0.02 cc. of filtrate shows after incubation confluent plaques.
Control 2	*Control 3*
Staph. Vnt. (heat-killed) 0.25 cc. incubated 35°C., 20 hours. Plaque test reveals no plaques. Staph. Vnt. added, incubated 35°C., 20 hours. Test-tube shows no evidence of lysis. Plaque test negative	0.05 cc. of Staph. Vnt. added to broth, incubated 35°C., 20 hours. Filtrate shows no evidence of plaques.

trates tested for ability to produce plaques. The method used and the results obtained are indicated in table 5.

When experiments of similar nature were repeated at 20°C. absorption of phage could also be demonstrated as indicated above, but when living culture was added complete lysis generally occurred in the test tube. The results of these and many other experiments of like nature show that bacteriophage absorbed upon heat-killed susceptible culture may under the influence of added living cells be freed. However, it is also possible that a very few corpuscles may have escaped absorption, and on the

addition of living cells proliferate, the result being the demonstration of plaques or lysis in the test tube. Though we have not been able to demonstrate it, it appears that test-tube evidence of lysis, under these conditions, depends on the rate of cell growth. At 20°C., the living staphylococci develop less rapidly than at 35°C. and the balance between cell multiplication and bacteriophage invasion by the freed phage is such that complete lysis takes place. Whereas at 35°C., while bacteriophagy does take place, as is evidenced by plaque demonstration, failure to clear in a broth tube culture is due to the higher rate of growth under these conditions. The bacteriophage development lags behind bacterial proliferation so that the end result is a tube that contains bacteriophage, although macroscopically it is similar to the bacterial control. Only plaque demonstration will show that bacteriophagy has occurred, under these conditions. It is also conceivable that at 35°C. the bacteriophage freed from the heat-killed culture may lack some of the types originally present in the unabsorbed phage, the result being failure of the tube to clear completely. A plaque method allows any and all types of phages to manifest themselves. Furthermore, if one or more types of the original phage are lacking the secondary culture which develops is always susceptible to the original complete phage. This may well represent a source of error in interpreting what a secondary culture is in terms of susceptibility or resistance to lysis.

Experiments were so conducted that although absorption of bacteriophage took place at 35°C., living culture was added and the tube incubated at 20°C. In these instances practically complete lysis took place in the test tube. Whereas if the absorption was carried out at 20°C. and the tube with added living culture incubated at 35°C., bacteriophagy was demonstrable only by the plaque method.

DISCUSSION

Working with staphylococci and staphylococcus bacteriophages one can prepare aqueous extracts of bacteria that will inhibit the action of bacteriophage. The ability of a given extract to inactivate a bacteriophage will depend largely on the susceptibility

of the culture furnishing the extract. With the cultures that we have examined, the most potent extracts were derived from highly susceptible bacteria. Extracts which act only in high concentrations have originated from cultures which are not completely lysed, reflecting probably a surface antigen, which so far as bacteriophagy is concerned is not homogeneous. That the composition of the surface antigen largely determines susceptibility or resistance to bacteriophage has been expressed by Burnet (1929) and the results with these staphylococcus extracts strengthens this viewpoint. Viewed somewhat differently the relation between bacterial extracts and phage bears out the early claims of d'Herelle that the first step in bacteriophagy is an absorption on the surface of the susceptible cell. It is probable that the susceptibility of other cells to other viruses has the same basis. Any change in the make-up of the surface antigen, one not necessarily as striking as the S → R, will result in a change in susceptibility toward a bacteriophage, and this change is accurately reflected in the capacity of the extract to inactivate phage. A susceptible culture gives rise to an extract that neutralizes bacteriophage, while a secondary culture made by subjecting this susceptible strain to a bacteriophage and therefore resistant to that phage, gives rise to an extract devoid of inactivating power. However, phages capable of lysing that secondary culture are also inhibited by the extract of that culture.

Strains of resistant bacteria can be isolated that can absorb phage in both the living and heat-killed states, without showing any evidence of bacteriophagy (such as strain Gec.), yet extracts prepared from this culture fail to inactivate phage. It may be that such uncommon strains of staphylococcus are composed of at least two surface components from the point of view of the bacteriophage, one susceptible, therefore capable of absorbing phage, the other resistant, the final result being a growth of the bacteria with the concomitant disappearance of the bacteriophage.

We feel with d'Herelle, Asheshov, Burnet and others that the best method for demonstrating bacteriophagy, or the effect of any agent on bacteriophage is one that entails plaque demonstration. The experience of several years with phages confirms the

belief that turbidity standards do not necessarily mean phage absorption or destruction, and that the demonstration of lysis against a few isolated colonies from such a culture is indicative that phage has not affected that culture. Asheshov (1933) has pointed out that in any phage-resistant culture (secondary) one can isolate colonies that are susceptible; it is only when the entire secondary culture is used that resistance to bacteriophage is consistently evident. Weak phages or a phage robbed of one or more of its types may never give rise to complete lysis in the test-tube, but with a plaque method macroscopic evidence of bacteriophagy is easily obtainable.

Our results with heat-killed cultures lead us to believe that absorption of bacteriophages by such means is not completely irreversible; that while the temperature at which the absorption is made to occur is of little importance, evidence of disunion of bacteriophage is obtained when the living cells are added and the experiment carried out at 20°C.

CONCLUSIONS

1. Susceptible cultures of staphylococci furnish extracts which inhibit bacteriophage.

2. There appears to be a relation between the susceptibility of a culture to phage and the ability of an extract of that culture to inactivate bacteriophage.

3. Extracts are heat-stable, being active after autoclaving.

4. Extracts lose their phage-inactivating capacity on filtering through L3, L5 Chamberland candles, Berkefeld and Seitz filters.

5. The phage-inactivating property of an extract is lost on precipitating with homologous anti-bacterial serum.

6. Heat-killed susceptible cultures of staphylococci absorb phage, as do the cultures after being autoclaved.

7. The union between bacteriophage and heat-killed culture under certain conditions appears to be reversible.

REFERENCES

ANDREWES, C. H., AND ELFORD, W. J. 1933 Brit. Jour. Exp. Path., **14**, 376.

APPLEBAUM, M., AND MACNEAL, W. J. 1931 Jour. Inf. Dis., **49**, 225.

ASHESHOV, I. N., ASHESHOV, I., SARANJAN, K., LAHIRI, M. N., AND CHATTERJI, S. K. 1933 Ind. Jour. Med. Research, **20**, 1101.

BAIL, O., AND OKUDA, S. 1923 Arch. f. Hyg., **92**, 251.
BURNET, F. M. 1930 Jour. Path. and Bact., **33**, 647.
BURNET, F. M. 1934 Jour. Path. and Bact., **38**, 285.
DACOSTA CRUS, J. 1922 Mem. Inst. Oswaldo Cruz, **14**, 81.
D'HERELLE, F. 1920 Compt. rend. Soc. de Biol., **83**, 97.
D'HERELLE, F. 1921 Le Bacteriophage: son rôle dans l'immunité. Paris, Masson et Cie, Monographe de l'Inst. Pasteur.
ELIAVA, G., AND POZERSKI, E. 1921 Compt. rend. Soc. de Biol., **84**, 708.
JAUMAIN, D., AND MEULEMAN, M. 1922 Compt. rend. Soc. de Biol., **87**, 362.
LEVINE, P., AND FRISCH, A. W. 1933a Proc. Soc. Exper. Biol. and Med., **30**, 993.
LEVINE, P., AND FRISCH, V. W. 1933b Proc. Soc. Exper. Biol. and Med., **31**, 46.
LEVINE, P., AND FRISCH, V. W. 1934a Jour. Exper. Med., **59**, 213.
LEVINE, P., AND FRISCH, V. W. 1934b Jour. Immunol., **26**, 321.
LEVINE, P., AND FRISCH, V. W. 1934c Proc. Soc. Exper. Biol. and Med., **32**, 341, 343.
PRAUSNITZ, C., AND FIRLE, E. 1924 Central. f. Bakt., 1 Orig., **93**, 158.
RAKIETEN, M. L. 1932 Yale Jour. Biol. and Med., **4**, 807.

THE INFLUENCE OF THE COMPOSITION OF THE MEDIUM ON THE METABOLISM OF SOME SLOW-LACTOSE-FERMENTING BACTERIA OF INTESTINAL ORIGIN

A. D. HERSHEY AND J. BRONFENBRENNER

Department of Bacteriology, Washington University Medical School, Saint Louis, Missouri

Received for publication, June 20, 1936

In the course of earlier studies (Bronfenbrenner and Schlesinger, 1918) of some slow-lactose-fermenting bacteria of intestinal origin, it was observed that the appearance of acid and gas in the lactose fermentation test was remarkably influenced by the composition of the medium.

The influence of buffer and of salts of organic acids upon sugar fermentation in general have been investigated by Clark (1915), and Ayers and Rupp (1918), respectively. The question has remained open, however, as to the specific manner in which the composition of the medium influences the development of the bacterial culture, and particularly, by what mechanism the concentration of lactose determines the rate of utilization of this sugar.

It was the object of the present investigation to determine whether the observed influence of concentration factors upon the fermentation of lactose by the slow fermenting strains was exerted merely upon the rate of growth of the organism, or upon its variability, or whether special phases of metabolism were primarily involved. It was necessary, therefore, to submit to separate analysis the several manifestations of growth and metabolism, utilizing various media of known composition.

The cultures studied consisted of a slow lactose-fermenting strain of *Escherichia coli*, and a rapidly fermenting variant derived from it. The isolation and characteristics of these

strains have been described elsewhere (Hershey and Bronfenbrenner, 1935).

The organisms were grown on a synthetic medium of the following composition: ammonium sulfate 0.5 gram, sodium chloride 0.5 gram, calcium chloride 0.005 gram, magnesium sulfate 0.02 gram, potassium acid phosphate 0.68 gram, dissolved in 100 cc. distilled water. Varying amounts of disodium succinate and of lactose were added to this basal medium as will be shown below. Sodium was used in preference to the ammonium salt of succinic acid so that the concentration of this acid could be varied independently of the nitrogen. For each experiment, 250-cc. amounts of the above-described medium were distributed in seven 1-liter Erlenmeyer flasks, and 10-cc. amounts in Dunham fermentation tubes of uniform size. These were sterilized under cotton plugs, and inoculated simultaneously from a twenty-four-hour agar culture. At this time the cotton plugs of two of the flasks were replaced by rubber stoppers equipped for aeration of the cultures. All were incubated at 37°C. The two sealed flasks were aerated daily for a period of two hours for the purpose of determining the amount of carbon dioxide produced. At intervals of two days one of the remaining cultures was centrifuged, after restoring the water lost by evaporation, and the supernatant fluid was used for analysis. *Succinic acid* was determined by titration of the precipitated silver salt (Volhard). *Lactose* was determined by the Schaffer-Hartmann micro method. Total *organic acids* were titrated by the method of Van Slyke and Palmer. The *hydrogen ion concentration* was determined colorimetrically. The amount of *growth* was measured gravimetrically after washing the centrifuged cells and drying to a constant weight. The amount of *gas* formed in the Dunham fermentation tubes was measured in linear centimeters. The *carbon dioxide* produced in the sealed flasks was absorbed into standard NaOH solution, precipitated as $BaCO_3$, and titrated by difference. Each culture was plated at the conclusion of its incubation period on a lactose China-Blue Rosolic-Acid medium (Bronfenbrenner 1918) to control purity and variation. The results plotted in figures 1 and 2 represent the means of two or more experiments.

THE INFLUENCE OF THE CONCENTRATION OF LACTOSE IN MEDIA CONTAINING LACTOSE AS SOLE SOURCE OF CARBON

In figure 1 are shown the changes brought about during the growth of a slow-lactose-fermenting strain in media containing lactose in concentrations of 0.5 and 3 per cent in the absence of any additional source of carbon.

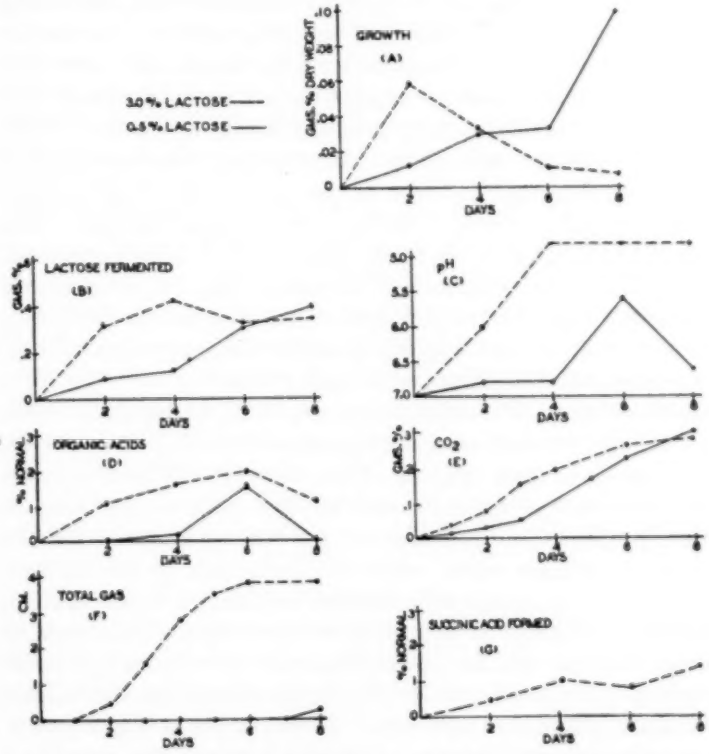


FIG. 1. INFLUENCE OF THE CONCENTRATION OF LACTOSE ON METABOLISM

Subcultures on the indicator plates, and in lactose fermentation tubes, revealed no increase in fermenting power during the growth of the cultures before the twelfth day of incubation. Bacterial variation, therefore, did not occur in either of these media during the course of the experiments.

The growth curves (fig. 1 A) show a more rapid development of the culture in the medium containing the higher lactose concentration, with an early interruption and subsequent autolysis of the non-aerated cultures. The flask examined at eight days was sterile, and almost water-clear.

Early lactose fermentation (fig. 1 B) was more rapid in the medium containing lactose in the higher concentration, but ceased with the development of high acidity. The apparent increase in lactose content of the medium between the fourth and sixth days can be due only to relative suppression of monosaccharide fermentation by the high acidity reached during this period, while activity continues unchecked. Accumulated monosaccharides liberated on cleavage of lactose by this enzyme would give nearly twice the copper reduction value of the original lactose. This increase was invariably observed when a pH of 4.8 was reached. During this period organic acid formation (fig. 1 D) was nearly stationary. Inhibition of glucolysis does not appear in the carbon dioxide curve (E) presumably because daily aeration of this culture prevented development of high acidity.

The hydrogen-ion concentration (fig. 1 C) and organic acids (fig. 1 D) also reached an earlier maximum value in the medium containing 3 per cent lactose. This effect should be attributed to the increased oxygen demand in this medium, resulting in relatively anaerobic conditions of growth unfavorable for the oxidation of organic acids, while acid formation by glucolysis is at its height. In the aerated cultures (not shown in the figures), although lactose was fermented even more rapidly, high acidity was not reached, and the development of the culture was uninterrupted. The accumulation of acid thus depends on the balance maintained between simultaneous fermentation of sugar and of the resulting organic acids, as first clearly stated by Ayers and Rupp (1918).

While the carbon dioxide curves were obtained from cultures not strictly comparable with those not subjected to aeration, they give, nevertheless, a direct measure of the relative rates of respiration in the two media. The effect of lactose concentration on carbon dioxide production (E) is slight compared with its effect

on removal of lactose (B). This is another expression of the limiting effect of oxygen supply on the rate of oxidation of the products of sugar cleavage, in contrast to the rate of cleavage itself.

Gas formation in fermentation tubes is primarily a measure of the ability of the organisms to develop anaerobically in a medium containing fermentable substances. It has no direct relation, therefore, to carbon dioxide production measured in aerobic cultures. The curves (F) showing gas formed in the Dunham fermentation tubes again illustrate the more rapid utilization of lactose when it is present in the higher concentration. Carbon dioxide : hydrogen ratios were roughly 1:2 in lactose media.

THE INFLUENCE OF THE CONCENTRATION OF LACTOSE AND OF SUCCINATE RESPECTIVELY IN MEDIA CONTAINING BOTH SOURCES OF CARBON

A second series of experiments was carried out, identical with those described above except that both lactose and sodium succinate were supplied in the basal medium. Two media were used, one containing 0.5 per cent lactose and 2.5 per cent succinate; the other 2.5 per cent lactose and 0.5 per cent succinate. These proportions were chosen to give extreme values for the ratio of lactose to succinate. Changes taking place during the growth of the slow lactose-fermenting strain in these media are shown in figure 2.

The first of the above represents an almost perfectly balanced medium. The curves obtained by analysis of the cultures are smooth throughout. The pH (fig. 2 C) remained practically unchanged during the course of the experiment, while the total amount of organic acids (D) exclusive of carbon dioxide was constantly decreasing. The rate of removal of succinate (G) was greater than the rate at which acids formed from the fermentation of lactose accumulated.

In the medium containing 2.5 per cent lactose and 0.5 per cent succinate, lactose fermentation, the formation of organic acids, and total gas production proceeded more rapidly than in the medium containing 0.5 per cent lactose and 2.5 per cent succinate.

This increase in the metabolic activity was similar in general to that observed in the absence of succinate (fig. 1). However, in the presence of the latter, growth (A) and fermentation (B) were

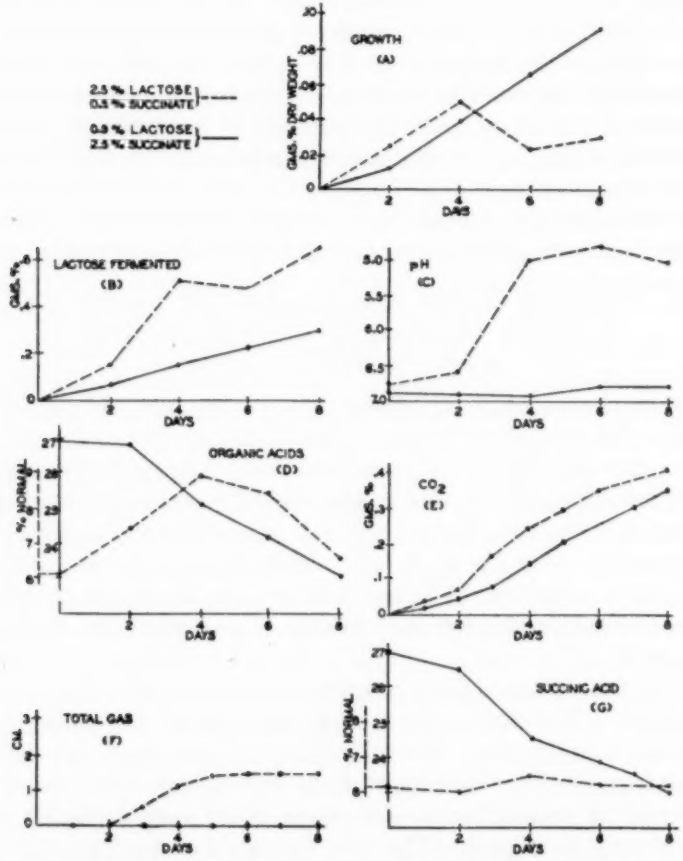


FIG. 2. INFLUENCE OF THE CONCENTRATIONS OF LACTOSE AND OF SUCCINATE ON METABOLISM

only temporarily suppressed by the accumulation of acid (D). The cultures survived, there was a reversion of pH (C) and fermentation of lactose was resumed. It can be seen that reversion

in this case was not due to exhaustion of the sugar, but apparently only to the decreasing rate of fermentation of lactose as its concentration diminished. The presence of succinate did not appear to affect lactose fermentation other than through its buffer-action favorable to survival of the culture. Differences in the respective curves showing lactose removal (B) in figures 1 and 2 are wholly referable to differences in the corresponding growth curves (A).

In the medium containing the higher concentration of lactose and the smaller amount of succinate, succinic acid removal was wholly suppressed (fig. 2 G). This may be attributed in part to the production of additional small amounts of this acid resulting from fermentation of lactose (fig. 1 G). Whether there is a direct effect of the concentration of succinate on its utilization is not clear from these experiments. Unquestionably considerations of oxygen demand are again pertinent here. In the more vigorously respiring culture, in which conditions of oxygen-starvation prevail, fermentation of succinate as well as of other aerobically fermented acids is inhibited. This mechanism, which has not been considered heretofore in this connection, perhaps defines the extent of the so-called protein-sparing action of fermentable sugar in bacterial cultures.

METABOLISM OF THE RAPIDLY LACTOSE-FERMENTING VARIANT

The following experiment, in which the behavior in a lactose medium of a rapidly fermenting variant is compared with that of the parent culture, illustrates several points in connection with the previous experiments. The synthetic medium already described, containing 0.5 per cent lactose and 2.5 per cent sodium succinate, was used. The curves are given in figure 3.

The rates of growth for the two strains as indicated in the curves (fig. 3 A) were nearly identical. For this reason the findings are particularly instructive. In the culture of the variant (V) strain, lactose (B) was completely removed by the fourth day, whereas twelve days were required in the case of the parent culture (P). In the more actively fermenting (V) culture, the removal of organic acids proceeded more slowly than their formation, resulting in the acid and alkaline phases (C) typical of bacterial

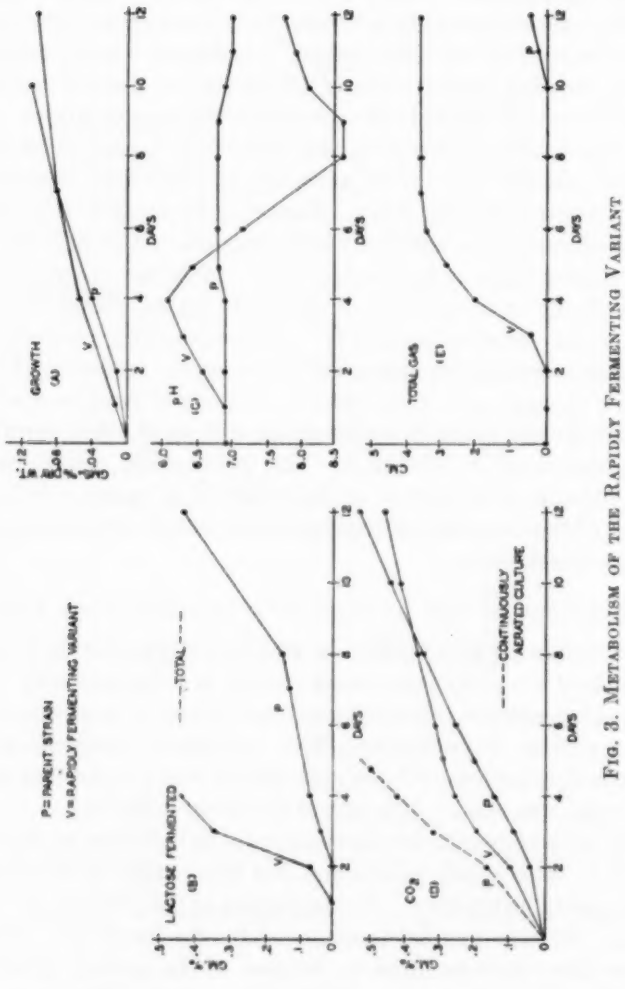


FIG. 3. METABOLISM OF THE RAPIDLY FERMENTING VARIANT

fermentation of sugars. Carbon-dioxide production (D) was somewhat more abundant in this culture, but the difference in respiration rate was much less than the difference in the rate of fermentation (B) and of total gas production (E). The essential difference between the two bacterial cultures is their content of the enzyme lactase, which is the limiting factor in the fermentation of lactose by these organisms (Hershey and Bronfenbrenner, 1935). Since, in the removal of lactose and to a large extent in the formation of gas in closed fermentation tubes, only the anaerobic processes of fermentation are concerned, it is in these two curves (B and E) that the difference between the metabolism of the two cultures is reflected. But only a small part of the carbon dioxide output of the aerated culture is derived from anaerobic processes, e.g., that represented by the equation: $HCOOH \rightarrow H_2 + CO_2$. The remainder is furnished by aerobic oxidations. Thus, the total carbon dioxide formed has two limiting factors: the rate of sugar fermentation, and the rate of respiration as determined by the oxygen supply. Accordingly the amount of carbon dioxide produced is somewhat greater in the rapidly fermenting culture than in the parent culture receiving the same amount of oxygen, but a much greater increase is obtained when the latter is continuously aerated (fig. 3 D). A similar discrepancy between the rates of fermentation (B) and respiration (E) appears in figures 1 and 2, as an effect of varying the concentration of lactose. There it is seen to be largely independent of the succinate supplied in the medium.

OXYGEN UPTAKE OF CELL SUSPENSIONS IN SOLUTIONS OF PURE LACTOSE

From the results of the foregoing experiments, it was not clear whether the effect of changes in the concentration of lactose in the medium was merely one of growth stimulation, with resulting accumulation of lactase, or whether there was a direct relation between the concentration of lactose and the rate of lactase activity. The following experiments were designed to answer this question by excluding the effect of growth stimulation. For this purpose the respiration of suspensions of washed cells of the

slow lactose-fermenting strain in lactose solutions of different concentration was measured during short periods of time under conditions in which multiplication of cells would be a negligible factor. To exclude the possibility that impurities in the lactose, or products of its decomposition during sterilization by heat, and not the concentration of lactose itself, were responsible for the effects observed (fig. 1), the oxygen uptake in unheated lactose solutions prepared from a twice recrystallized preparation of the sugar was controlled by a parallel experiment using autoclaved solutions of commercial lactose. All substrates were dissolved in M/20 phosphate buffer of pH 7.0. The measurements were made in the Barcroft apparatus at 37.5°C., in vessels designed to permit

TABLE 1

Effect of concentration on oxidation of lactose

SUBSTRATE	O_2 UPTAKE	PER CENT INCREASE
	cm. per 10 minutes	
Purified lactose:		
Initial concentration, 0.5 per cent	0.30; 0.35; 0.35	150
Final concentration, 2.5 per cent	0.77; 0.84; 0.78	
Autoclaved commercial lactose:		
Initial concentration, 0.5 per cent	0.31; 0.33; 0.34	150
Final concentration, 2.5 per cent	0.83; 0.80; 0.75	

the addition of substrate during the course of the experiment. The cell suspension was placed in the vessel with an initial substrate, and three consecutive readings were made at ten-minute intervals. Then the new substrate was admitted, and additional readings taken in the same manner. Table 1 shows the relation between concentration of the two samples of lactose, and oxygen uptake. Manometer values are expressed in linear centimeters. It may be seen that there is a direct relation between the concentration of lactose and the oxygen uptake, and that the values for purified unheated lactose solutions are identical with those for the autoclaved commercial preparation.

As a further control on the preceding experiment, the effect on the rate of respiration in the presence of lactose of the addition

of a small quantity of glucose (comparable to the amount of monosaccharides which might be liberated by hydrolysis during sterilization) was measured. This is shown in table 2.

Here it is seen that the stimulation by small amounts of monosaccharide is of greater magnitude, and of shorter duration, than that provided by increased concentration of lactose alone. It is improbable, therefore, that the influence of the concentration of lactose in the preceding experiments can be attributed to products of its decomposition by heat.

TABLE 2

Effect of added glucose on the oxygen uptake in lactose

SUBSTRATE	O_2 UPTAKE	PER CENT INCREASE
	cm. per 10 minutes	
Purified lactose, 0.5 per cent	0.57; 0.62; 0.59	700, rapidly decreasing
Added glucose, 0.02 per cent	6.9 ; 4.8 ; 2.8	

TABLE 3

Growth and fermentation in purified lactose media

MEDIUM	0 DAY	1 DAY			2 DAYS			3 DAYS			4 DAYS		
	pH	pH	Growth	Gas	pH	Growth	Gas	pH	Growth	Gas	pH	Growth	Gas
0.5 per cent lactose, unheated	6.8	6.8	1	0	7.0	2	0	6.8	2	0	6.8	2	0
3.0 per cent lactose, unheated	6.8	6.8	2	0	5.6	3	5	5.0	4	17	4.8	4	20
3.0 per cent lactose, autoclaved	6.6	6.6	2	0	5.6	3	12	5.0	4	17	4.8	4	20
0.5 per cent lactose, unheated, plus													
Glucose 0.01 per cent	6.8	6.8	2	0	6.8	2	0	7.0	3	0	7.0	3	0
Glucose 0.10 per cent	6.8	6.8	2	0	6.8	3	0	6.6	3	0	6.5	4	0
Glucose 0.20 per cent	6.8	6.6	2	5	6.6	3	6	6.5	4	6	6.4	4	6

FERMENTATION AND GROWTH IN PURE LACTOSE MEDIA

Experiments with the bacterial suspensions showed clearly that the fermentative activity of the cells is dependent on the concentration of lactose itself. It was thought desirable to re-examine also the effect of concentration on growth, using purified, unheated lactose. Fermentation tubes containing

different amounts of lactose, with or without added glucose, and 1 per cent peptone, M/20 phosphate buffer, and China-Blue Rosolic-Acid indicator (Bronfenbrenner, 1918) were prepared. One series of tubes were autoclaved, in another, filtered solutions of purified lactose were added to the sterile medium. Both sets were inoculated with the slow fermenting strain, and observed daily for comparison of turbidity, pH, and total gas. Results are shown in table 3. It is again evident that the early growth and fermentation of the slow fermenting strain in lactose media is a function of lactose concentration, and that this effect is not traceable to impurities or to products of the decomposition of lactose incidental to sterilization of the medium by heat.

SUMMARY

1. The metabolism of a slow-lactose-fermenting strain of the *Escherichia coli* type, and of its rapidly fermenting variant, in lactose-containing synthetic media, have been studied. Figures are given showing the development of the cultures and the accompanying metabolic changes as they occur in these media in relation to the lactose concentration, and to the presence of an additional source of carbon.

2. Variation within the culture does not play any part in the early course of fermentation by these bacteria.

3. The multiplication rate, and the velocity of lactose fermentation, are markedly influenced by the concentration of lactose in the medium. The effect on the rate of fermentation is direct, as well as dependent on changes in the rate of growth.

4. The rate of fermentation varies with the bacterial population of the culture, while the rate of respiration is largely independent of it. This divergence between the rates of fermentation and respiration is discussed without reference to "phases of growth."

5. The presence of sodium succinate as an accessory source of carbon does not appear to influence early lactose fermentation, except through its buffer-effect on the pH of the culture.

6. Succinic acid is removed from the medium more rapidly as

the ratio of the concentration of succinic acid to that of lactose is increased.

REFERENCES

AYERS, S. H., AND RUPP, P. 1918 Jour. Inf. Dis., **23**, 188.
BRONFENBRENNER, J. 1918 Jour. Med. Res., **39**, 25.
BRONFENBRENNER, J., AND SCHLESINGER, M. J. 1918 Proc. Soc. Exp. Biol. and Med., **16**, 44.
CLARK, W. M. 1915 Jour. Biol. Chem., **22**, 87.
HERSHEY, A. D., AND BRONFENBRENNER, J. 1935 Jour. Bact., **31**, 451.

SOME CHEMICAL FACTORS INFLUENCING GROWTH AND PIGMENTATION OF CERTAIN MICROÖRGANISMS[1]

M. S. KHARASCH, E. A. CONWAY AND W. BLOOM

Contribution from The George Herbert Jones Chemical Laboratory and The Anatomy Department of the University of Chicago

Received for publication July 8, 1936

This is a report on some observations regarding the effects of metals and other substances on the growth and pigmentation of bacteria. A brief explanation of our interest in this field will enable the reader to follow the general trend of our thesis. In a contribution from this laboratory (Kharasch, Legault, Wilder and Gerard, 1936) the factors which govern the oxidation of certain thiol systems to the disulfides have been evaluated. The thiol systems generally are extremely sensitive to traces of heavy metal ion catalysts, particularly those of manganese (Mn^{++}), copper (Cu^{++}), and iron (Fe^{+++}). The effectiveness of these ions as catalysts varies widely. Manganese is catalytically the most effective, while iron is weakest and copper intermediate.

It was further demonstrated that the catalytic effects of the metal ions, particularly those of copper and iron, could be inhibited by the addition of various extracts to the system. Thus, beef extract was found to inhibit the catalytic effect of iron, but not that of copper or manganese, in the oxidation of thiols by oxygen. Liver extract on the other hand, was found to inhibit powerfully the catalytic effect of both iron and copper ions, but not that of the manganese ion. The inhibition of copper ion catalysis in thiol systems to which liver extracts had been added was most striking. Furthermore, chicken liver extracts on a

[1] This investigation was aided by a grant from the Rockefeller Foundation to the University of Chicago. We are indebted to Miss V. Oelgeschlager for aid in some of the preliminary experiments.

dry-weight basis appeared to contain more of the specific copper inhibitor than beef or calf liver extracts.

A simple working hypothesis of the effectiveness of these extracts in the inhibition of metal catalysts of thiol systems is that these extracts contain substances which form, with the metal ions, complexes that are dissociated to such a slight extent that the relative number of free metal ions is reduced tremendously.

These experiments, and the tentative hypothesis advanced to explain the inhibitory effect of liver extracts on copper catalysis, suggested the extension of the experiments to the study of the growth of *Chlorella vulgaris* in a medium containing copper and liver extract. *Chlorella*, as is well known, is extremely sensitive to even minute traces of copper. An exceedingly interesting biologic experiment was thus available to test our hypothesis. We realize of course, the essential limitations of the hypothesis, and the possibility of other interpretations of the data. But we believe that the hypothesis advanced by us to account for the effectiveness of liver extracts in suppressing the catalytic activity of copper is the most satisfactory suggested by the data at present available, and is furthermore corroborated by additional evidence from other systems.

Our first experiments were made on a strain of *Chlorella vulgaris* cultured in a synthetic medium (Meier, 1932) of inorganic salts, a small amount of arginine and plain agar. *Chlorella* normally appears as a green growth on this basic medium. Our experiments confirmed the observations of others that *Chlorella* does not grow on this medium or on nutrient agar in the presence of minute amounts of copper. However, when liver extract was added to such a copper-containing medium, there was an abundant growth, but, strikingly, the color was not the characteristic green, but a dark orange. When such cultures were examined microscopically, it was found that the relatively large green alga had changed into a small spherical body, slightly larger than the staphylococcus. This is the form which *Chlorella* is assumed to take under adverse conditions (dehydration, etc). The protective action of liver extracts against the toxic effects of copper was definitely demonstrated.

The change in morphology and pigmentation of *Chlorella* under the influence of copper and liver extract naturally suggested a similar study with bacteria. Investigation of the effect of addition of the biologically important metal ions (Fe^{+++}, Cu^{++}, Mn^{++}), to usual media on the growth and the pigmentation of bacteria, as well as possible "detoxification" of the metals by liver extracts, was undertaken.

Table 1 brings out a number of interesting facts. In the first place, it indicates a distinct difference in tolerance of the different organisms toward metals. Thus, both copper and manganese in dilutions of 1:50,000, when added to plain agar, inhibit the growth of *Spirillum rubrum*, but have little or no effect on the growth of the other organisms. Iron, on the other hand, even in a concentration of 1:10,000 enhances rather than retards growth of the organisms tested. Second, the effect of the metals on bacterial pigmentation is of interest. Iron added to the medium has no marked effect on pigmentation and, if anything, augments it. In the presence of manganese, however, *Staphylococcus aureus* is not pigmented, while the pigmentation of the other organisms is either unaffected or augmented. The addition of copper to the medium does not affect the growth of *Staphylococcus aureus* and *Pseudomonas pyocyanea*, but the growth is colorless. In the presence of glucose copper increases the intensity of the pigment in *Torula rosea*.

The addition of liver extract to the media in general had a beneficial effect upon the growth of the bacteria, and in no case did the extract appreciably inhibit bacterial pigmentation or markedly change the color, although the shade was altered in some instances. The effects of the addition of liver extract to media containing the various metals are included in table 1. In these preliminary experiments, however, our efforts were confined to a detailed study of *Staphylococcus aureus* only. No effort was made in the other cases to ascertain the optimum concentration of the copper and manganese content of the media. The metal concentrations employed throughout our experiments were based upon our experience with *Staphylococcus aureus*, and, consequently no undue importance should as yet be attached to the

TABLE 1

Effect of metals alone and with liver extract on growth and pigment formation

ORGANISM	NUTRIENT AGAR	NUTRIENT AGAR GLUCOSE, 1 PER CENT	NUTRIENT AGAR LIVER EXTRACT,* 0.8 PER CENT	NUTRIENT AGAR, Cu 1:50,000	GLUCOSE NUTRIENT AGAR, Cu 1:50,000	NUTRIENT AGAR LIVER EXTRACT,* Cu 1:50,000	NUTRIENT AGAR, Fe 1:10,000	NUTRIENT AGAR LIVER EXTRACT,* Fe 1:10,000	NUTRIENT AGAR, Mn 1:50,000	NUTRIENT AGAR, LIVER EXTRACT,* Mn 1:50,000
Staphylococcus aureus...	Dull orange	Dull orange	Dull orange	Non-pigmented		Dull orange	Dull orange	Dull orange	Non-pigmented	Non-pigmented
Pseudomonas pyocyanea.....	Green	Green	Green	Non-pigmented		Non-pigmented	Green	Green	Green	Green
Serratia marcescens.....	Non-pigmented	Red	Orange-red		Red	Blue-red	Orange-red	Red	Pale red	Orange-red
Torula rosea.....	Non-pigmented	Rose-pink	Pale pink		Intense rose-pink	Rose-pink	Faint rose	Rose-pink	Pale pink	Rose-pink
Sarcina lutea.....	Yellow	Bright yellow	Yellow	Yellow		Yellow. Poor growth	Deeper yellow than normal	No growth	Yellow	Yellow
Spirillum rubrum.....	Faint pink	Blue-red	Pale red	No growth		No growth	Blue-red	No growth	Red. Poor growth	No growth
Aspergillus niger.....	Black	Black	Black	Black		Black	Black	Black	Black	Black. Slow developing

Nutrient agar: 3.5 grams beef extract; 10 grams peptone; 20 grams granular agar (all Difco); 5.5 grams sodium chloride C.P.; 1000 cc. distilled water. pH 7.4. Sterilised by autoclaving 15 minutes at 15 pounds pressure.

* Both chicken and beef liver extracts were employed, and gave concordant results. For preparation see reference 1.

results with the other organisms. Our general conclusion we believe to be thoroughly justified because of the effect of metals in general on pigmentation. Naturally, there would be an optimum effective metal concentration for each organism, and due caution must be exercised in a general statement until this study has been completed. Yet our results, although confined to *Staphylococcus aureus* only, are of interest, and in excellent agreement with our general thesis. Thus, with high concentrations of copper on beef infusion agar no growth was obtained. Similar media, however, to which liver extract was added resulted in a colorless growth. With low concentration of copper on the same medium a colorless growth of *Staphylococcus* was obtained. The addition of liver extract, however, to this medium containing low concentration of copper produced a good growth of pigmented *Staphylococcus aureus*. It is of interest that the addition of liver extract to a medium containing low concentration of manganese was ineffective in restoring the color to the non-pigmented *Staphylococcus*. This observation is in agreement with our previously cited cases, to the effect that liver extracts are exceedingly effective in overcoming the effects of copper and iron but do not inhibit the effects of manganese.

The fact that in the presence of metals some ordinarily pigmented bacteria grow without producing a pigment as in the case of *Staphylococcus aureus*, *Pseudomonas pyocyanea*, or to a different color as in the case of *Chlorella*, naturally raised the question whether some reagent or reagents could be found which would inhibit pigmentation in all of the organisms we have been working with, without interfering with their growth.

Table 2 indicates that in diphenylamine we have a remarkable substance which produces a colorless growth of all of the organisms we have investigated (with *Aspergillus niger* pigment production was delayed). The medium apparently is of minor importance to the extent that glucose agar and nutrient agar containing various added substances, media which in the case of all the organisms give normal growth and pigmentation, give rise to only colorless forms when quantities of diphenylamine of the order 1:2000 or 1:6000 are added.

TABLE 2

Effect of diphenylamine and of substituting aldehydes and ketones for glucose on growth and pigment production

	GLUCOSE AGAR DIPHENYLAMINE	NUTRIENT AGAR GLUTATHIONE, 1:2000	NUTRIENT AGAR DIPHENYLAMINE GLUTATHIONE	NUTRIENT AGAR ALLOXANTIN, 1:2000	NUTRIENT AGAR DIPHENYLAMINE ALLOXANTIN	NUTRIENT AGAR VERATRALDEHYDE, 1:2000	NUTRIENT AGAR DIPHENYLAMINE VERATRALDEHYDE	NUTRIENT AGAR VIOLURIC ACID, 1:2000	NUTRIENT AGAR DIPHENYLAMINE VIOLURIC ACID
Staphylococcus aureus	Non-pigmented	Dull orange	Non-pigmented	Dull orange	Non-pigmented	Deeper orange than normal	Non-pigmented	Dull orange	Non-pigmented
Pseudomonas pyocyanea	Non-pigmented	Green	Non-pigmented	Green	Non-pigmented	Non-pigmented	Non-pigmented	Medium decolorized	Medium decolorized non-pigmented
Serratia marcescens	Non-pigmented	Red pigment after 5 generations	Non-pigmented	Red after repeated transfer	Non-pigmented	Pink color increased with each transfer	Non-pigmented	Red	Non-pigmented
Torula rosea	Non-pigmented	Rose pigment in 3d generation	Non-pigmented	Rose after repeated transfer	Non-pigmented	Growth retarded after 3d generation	Poor growth	Rose color increased with each transfer	Non-pigmented
Sarcina lutea	Non-pigmented	Growth and yellow pigment greater than on nutrient agar	Non-pigmented	Yellow	Non-pigmented	Deeper yellow than normal	Non-pigmented	Poor growth	Poor growth
Spirillum rubrum	Fair growth non-pigmented	Some growth faint red	Poor growth, rose	Fair growth, red	Poor growth non-pigmented	Poor growth	Poor growth	Poor growth	Poor growth
Aspergillus niger	Fair growth, black pigment appears 4th day	Dense black growth	Poor growth, black pigment barely perceptible	Black	Poor growth	Dense black growth	Poor growth, black pigment formation delayed and decreased	Black pigment of same concentration as with glucose agar	Black pigment decreased

Some experiments recorded in table 2 were designed to demonstrate that specific groups rather than specific molecules are essential for bacterial growth and pigmentation. Thus, it is well known that *Serratia marcescens* rarely produces color on plain agar in the absence of glucose. Accordingly, glucose was replaced by other substances containing aldehyde or ketone groups. As may be seen from table 2 such substances as violuric acid, veratraldehyde, glutathione, alloxantin, and even Michler's ketone (tetramethyldiaminobenzophenone $(CH_3)_2NC_6H_4)_2{:}CO$) could be substituted for glucose without impairing growth or pigmentation. It is true, as was to be expected, that in each instance the pigment was slightly different from the characteristic carmine-red, and the bacteria had to become accustomed to the substance which replaced the glucose in the medium so that the deepest colors were obtained only after transplanting onto the same medium three to five times. In the end, however, pigmented *S. marcescens* which varied from a yellow-orange to a purple-blue was obtained. There is little doubt that with the appropriate selection of the aldehyde or ketone almost any shade of *S. marcescens* may be obtained.

Table 2 should also be consulted regarding the effect of the substances used in replacing glucose in the nutrient agar medium on the growth and pigmentation of organisms other than *S. marcescens* and *Torula rosea*, although the others do not depend upon the presence of glucose for their pigmentation.

SUMMARY

1. Large concentrations of biologically important catalytic metals (Mn^{++}, Cu^{++}, Fe^{+++}) inhibit growth of microörganisms, while smaller concentrations lead in some cases to loss of pigmentation.

2. Where the organisms are very sensitive to copper or when pigmentation is inhibited by it, the toxic effect of the metal may be counteracted by the use of liver extracts.

3. The binding effect of metals by liver extracts leads in the case of organisms which require metals, particularly iron, for their growth, to an unusual, but easily understandable, result, namely,

inhibition of growth by liver extract. *Sarcina lutea* is the most striking example of the type of organisms where growth is inhibited by liver extracts.

4. The development of pigment in cultures of *Serratia marcescens* does not specifically require glucose in the medium but depends upon the presence in it of substances which contain available aldehyde or ketonic groups.

5. A striking fact was brought out, namely, that pigment formation in *Staphylococcus aureus*, *Pseudomonas pyocyanea*, *Serratia marcescens*, *Torula rosea*, *Sarcina lutea*, *Spirillum rubrum*, and *Aspergillus niger*, when grown in different media could be inhibited by diphenylamine. In some instances the loss of pigment could be attained on the medium containing the diphenylamine on the first transplant, while others required several subculturings.

6. The work is being continued.

REFERENCES

KHARASCH, LEGAULT, WILDER AND GERARD 1936 J. Biol. Chem., **113**, 2, 537–569.

MEIER, F. E. 1932 Lethal action of ultra-violet light on a unicellular green alga. Smithsonian Miscellaneous Collections, **87**, 1.

STUDIES ON HEMOLYTIC STREPTOCOCCI

III. STREPTOCOCCUS EQUI AND RELATED STRAINS

ALICE C. EVANS

Senior Bacteriologist, National Institute of Health, Washington, D. C.

Received for publication July 2, 1936

CHARACTERS OF STREPTOCOCCUS EQUI AS DESCRIBED IN THE LITERATURE

It was not until 1887 that a distinction between the streptococcus of strangles and other streptococci was recognized, although streptococci had previously been regarded as the causal agent of strangles (Brocq-Rousseu gives a review of the early history of the disease).

In a footnote in Friedberger and Fröhner's *Lehrbuch der Speciellen Pathologie und Therapie der Hausthiere*, the authors state that they received a communication from Schütz which they quote verbatim. It describes the streptococcus of strangles so briefly that the entire footnote may be quoted here.

> Der ursächliche Erreger der Druse ist ein kettenbildender Micrococcus (Streptococcus), der sich in Ausstrichpräparaten des Lymphdrüseneiters nach Färbung mit Anilinfarben, z. B. Gentianaviolet, leicht nachweisen lässt. Er wächst nicht in Fleischwasserpeptongelatine, auch nicht in Agar-Agar. In Fleischinfusen bildet er eine flockige Masse am Boden der Gefässe und auf erstarrtem Hammelserum glasige, durchsichtige Tropfen, die später confluiren. Im Tropfen der Serumgläser entstehen dabei graue wolkige Massen. Er wirkt auf Mäuse pathogen, ruft an der Impfstelle Eiterung hervor und bedingt metastatische Processe auf dem Wege der Lymph- und Blutbahn. Der Drusencoccus stimmt mit keinem der bisher bekannten überein, namentlich nicht mit Streptococcus pyogenes, der bekanntlich in Fleischwasserpeptongelatine und in Agar-Agar die Bedingungen für sein Wachsthum findet.

In this original description Schütz mentions one character of the streptococcus of strangles—failure of growth in ordinary media—which differentiates it from other streptococci. In the study of the strains of hemolytic streptococci of our collection which now number about 600 strains, Schütz's observation of this distinguishing character was confirmed. The strains, which for reasons to be discussed further on, must be regarded as typical *Streptococcus equi* grew poorly or not at all in plain broth. The only other group of strains of our collection which exhibited this character was a small group of human origin received from Doctors Long and Bliss, representing their collection of "minute hemolytic streptococci." In their original description of these strains they commented on the resemblance to *S. equi*. The strains of Long and Bliss are readily distinguishable from *S. equi*, however, by their phagological reactions, and by other characters. Another character mentioned in Schütz's original description—the development of watery, confluent colonies on coagulated serum, is not distinctive, for it is a character of other groups of hemolytic streptococci.

In the following year Schütz published a more complete description of the streptococcus of strangles. He stated that of the many cases of strangles he had occasion to study, the organism which he describes was cultivated from all. He inoculated it into horses and described the symptoms and pathology of the resulting disease. He noted the high degree of pathogenicity of this organism for white mice, a character which has been repeatedly confirmed by later investigators.

Shortly after Schütz's publication of the original description of the streptococcus of strangles, and a few months before his complete description was published, Poels of Rotterdam and Sand and Jensen of Copenhagen published descriptions of the streptococcus of strangles. (Sand and Jensen also give a history of strangles.) In their summary Sand and Jensen designate the causal organism as *Streptococcus equi*. This seems to be the first use of that name. Their description of the streptococcus of strangles differs from Schütz's in that they sometimes obtained growth in ordinary media.

Another distinguishing character of *S. equi* seems to have been first noted by Piorkowski (1902). His observation that *S equi* is characterized by inability to produce a high titer of agglutinins in the serum of inoculated animals, has been confirmed by a number of investigators. Schnürer could not demonstrate agglutinins in the serum of horses, donkeys or rabbits treated with *S. equi*. Stolpe treated a sheep with *S. equi* and obtained serum with a titer of only 1:50. Pricolo found that the serum of horses treated with *S. equi* failed to agglutinate the homologous streptococcus. Van Straaten also failed to demonstrate agglutinins for the strangles streptococcus in the serum of treated horses. Brocq-Rousseu treated three horses with different strains of *S. equi*. In one, the titer of agglutinins for *S. equi* was 1:25, in another, 1:50, but in the third it was 1:800. In all probability the strain which produced the highest titer of agglutinins was not typical *S. equi*, for, as it will be shown further on, atypical strains of *S. equi* may produce agglutinins.

Schnürer reported that he found *S. equi* incapable of producing a protective serum. He found a total lack of protection of white mice against *S. equi* by the serums of spontaneously immunized horses, or by the serums of experimentally immunized rabbits and a donkey. On the other hand, Aronson immunized horses with the streptococcus of strangles, and obtained from them a protective serum. Pricolo also demonstrated immunizing action in the serum of a horse experimentally treated with the streptococcus of strangles. Our experiments, reported further on, agreed with those of Aronson and Pricolo in demonstrating a protective action in the serum of rabbits immunized with *S. equi*.

It was not until 1911 that another significant distinguishing character of *S. equi* was described. Jensen then reported the unpublished observation previously made by Holth, that the streptococcus of strangles differs from that which causes equine influenza in its inability to ferment certain test substances. Later investigators confirmed Holth's observation, but found that most of the 43 test substances which he used had no differential value. Ogura pointed out the usefulness of lactose, trehalose

and sorbitol. Edwards confirmed and amplified Ogura's observations.

There has been a disagreement among investigators as to whether the streptococcus of strangles is capable of fermenting lactose. Some assert that it lacks that capacity (Holth; Adsersen; Jones; Holman; Haupt; Ogura; and Edwards). On the other hand many investigators have reported that some or all of their strains from strangles were able to ferment lactose or produce a red color in litmus milk (Schnürer; Todd; Koch and Pokschischewsky; Maass; Sande; Geweniger).

A few investigators have considered the fermentation of sorbitol by the streptococcus of strangles and found it negative (Holth; Adsersen; Ogura; Edwards). On the other hand Koch and Pokschischewsky reported that some of their strangles strains fermented sorbitol. Holth, Ogura, and Edwards considered also the fermentation of trehalose and found it negative.

A number of investigators have tested the virulence of the streptococcus of strangles for a variety of experimental animals. Some of them report that it is virulent for horses and white mice, but that it is non-virulent for other animals (Sand and Jensen; Schütz; Schnürer; Straaten; Ogura). Other investigators report that the streptococcus of strangles is virulent for host species other than horses and white mice. Thus Poels reported that the streptococcus of strangles is pathogenic for guinea pigs and rabbits; Stolpe reported several human cases contracted from horses with strangles; Pricolo reported that the streptococcus of strangles is pathogenic for sparrows; Brocq-Rousseu reported that 7 out of 12 strains were pathogenic for guinea pigs.

According to the foregoing review there are in the literature many discordant reports of the characters of *S. equi*. An explanation which will account for the discrepancies will be given in the discussion at the end of this paper.

SOURCES OF S. EQUI STRAINS

The 20 strains of *S. equi* included in this study were received from a number of investigators in response to requests for cultures isolated from strangles in horses. The writer acknowledges

TABLE 1

Histories of the strains of Streptococcus equi

STRAIN NUMBER	PREVIOUS DESIGNATIONS	BY WHOM ISOLATED	LOCATION OF THE DISEASED HORSE	OTHER INVESTIGATORS
725	D1 K155B	Dr. P. R. Edwards, Lexington, Ky.	Kentucky	Dr. Rebecca C. Lancefield
727	D3			
939	K196			Dr. Lancefield
726	D2	Dr. F. S. Jones, Princeton, N. J.	?	Dr. Edwards
865	D10 02451	At the laboratories of Parke, Davis and Company, Detroit, Mich.	The horses were received from Illinois, Nebraska or Iowa	Dr. Edwards
866	D11 02493			
867	D12 02527			
868	D13 02537			
969	D14 02538			
870	D15 02648			
871	D16 02660			
930	742–3	Dr. Robert Graham, Urbana, Ill.	Illinois	
940	418	Dr. Axel Thomson, Copenhagen, Denmark	Denmark	
941	419			
942	420			
944	417			
1225	1	Maj. F. H. K. Reynolds, Army Medical Center, Wash., D. C.	Front Royal, Va.	
1226	2			
1237	89F8		Fort Hoyle, Md.	
1238	33F3			

indebtedness to all of those who responded. In order that the records of the strains may be complete, the history of each strain, in so far as it is known, is given in table 1.

THE TYPE STRAIN

Strain 725 was chosen as the type strain. It has been deposited in the American Type Culture Collection.

Strain 725 was isolated in January, 1931, by Dr. P. R. Edwards, from an abscess of a submaxillary lymph gland in a yearling colt with strangles. The differential characters of strain 725, determined according to the methods described in the first paper of this series, are as follows:

It is sensitive to phages B/563, C/594, C/646, and D/693 in the nascent state; it is sensitive to B/563 filtrate. Salicin is fermented; lactose, mannitol, trehalose and sorbitol are not fermented.

Other characters of strain 725 are as follows: There is no growth on 10-per-cent bile blood agar; sodium hippurate is not hydrolyzed; growth in plain infusion broth is scanty or lacking; in serum broth growth occurs rapidly at 37°, and slowly at 20°C. Human fibrin is not lysed; under the conditions of the test, final pH in glucose broth was 5.2.

On blood agar after 24 hours incubation the colonies are very small; after 48 hours the isolated colonies are 1.5 mm. or more in diameter, and adjacent colonies are watery and confluent. There is rapid drying out of the watery growth leaving thin, flat, matt colonies. Hemolysis is strong, resulting in a wide clear zone.

Smears from a 24-hour serum broth culture stained with Gram-safranin showed diplococci, some in short chains of about 4 cells, with an occasional chain of moderate length. The stain was retained only slightly.

THE VIRULENCE OF THE TYPE STRAIN FOR EXPERIMENTAL ANIMALS

The virulence of strain 725 for mice is high, with doses of 0.00000001 cc. of a passage strain regularly fatal.

The virulence of strain 725 for rabbits was tested in 4 animals. Inoculations were made with overnight blood-broth culture planted with the stock culture. Two out of three mice inoculated with 0.0000005 cc. of this culture died within four days.

Two rabbits were inoculated intravenously with 0.01 cc. of the culture, and having shown no symptoms of infection, they were reinoculated two days later with 0.1 cc. of culture. One of these rabbits (no. 225) showed no evidence of illness. During the six weeks following the inoculations the temperature was observed daily. It never rose above 39.6°C. except once, on the third day after the second inoculation, when it rose to 40.2°C. The other rabbit of this lot (no. 254) developed a chronic disease. Following the second inoculation the temperature varied between 40.2° and 41.2°C. for about three weeks, when an inflammation of the eyes and difficulty in breathing developed. Death occurred on the twenty-eighth day after the second inoculation. There were necrotic areas in the lungs and a purulent effusion in the pleural cavity. A blood agar culture from the heart blood yielded no streptococci, and only a few scattered colonies of an extraneous organism. In this case, although the inoculated organism was not recovered, the prompt development of chronic disease following inoculation, and the failure to demonstrate any other infection, suggests that the disease may have been the result of inoculation with *S. equi*. The chronic form of the disease in this animal agrees with Van Straaten's observation that the streptococcus of strangles causes a chronic, fatal disease in rabbits.

Two rabbits of another lot were inoculated with 0.1 cc. of culture 725, and having shown no symptoms of infection, they were reinoculated two days later with 1.0 cc. of culture. One of these animals (no. 264) showed no evidence of illness and failed to develop an abnormal temperature during a month's observation. Following the inoculation the other rabbit of this lot (no. 265) developed an abnormal temperature which varied between 40° and 41°C. for about seven weeks, when the temperature returned to normal and the rabbit appeared well. About a week later, however, it was found to be partially paralyzed.

Death occurred the following day. At autopsy no evidence of disease could be found, except that a cluster of larval tapeworms (*Cysticercus pisiformis*) were found attached to the omentum. A culture from the heart blood remained sterile. In this case it is questionable to what extent the inoculation with *S. equi* was responsible for the illness of the animal.

The results of the four experiments in which rabbits were inoculated with large doses of *S. equi* showed that it is only slightly virulent for these animals, producing a chronic disease, or none.

The virulence of strain 725 for guinea pigs was tested in 4 animals. Inoculations were made with overnight blood-broth culture planted with the stock culture. The virulence of the culture for a series of mice was tested with the following results: Mice receiving 0.001 and 0.00001 cc., respectively, died, and the streptococcus was recovered from the heart blood; the mice receiving 0.0001 and 0.000001 cc., respectively, survived.

The 4 guinea pigs each weighing about 530 grams were inoculated intraperitoneally with 0.1, 0.01, 0.001 and 0.0001 cc. of culture, respectively. Three of the animals remained well and continued to increase in weight during two week's observation. The animal which received 0.001 cc. of culture died on the eleventh day, and a Gram-negative rod was cultivated from the heart blood.

The results of the guinea-pig inoculations show that this species of animals may withstand inoculation with *S. equi* without disturbance of health.

PRODUCTION OF AGGLUTININS BY THE TYPE STRAIN

Rabbits were treated with the type strain (725) and with the atypical strain 865 (to be discussed further on) to determine their ability to produce agglutinins. Strains of two other species were tested for ability to produce agglutinins in parallel experiments. Three rabbits were treated with each strain, in order that individual peculiarities might not confuse the results.

The rabbits each received about 24 cc. of broth culture killed at 56°C. for one hour. Given in 14 doses at three- or four-day intervals, the type strain failed to produce agglutinins in all 3 rabbits, thus agreeing with the descriptions of *S. equi* already

quoted, whereas the streptococci of the other two species produced agglutinins in titers of 1 to 8000 or higher. Streptococcus 865, representing the atypical strains of *S. equi* was intermediary in its ability to produce agglutinins, giving titers of about 1 to 800 in each of the 3 rabbits.

PRODUCTION OF A PROTECTIVE SERUM BY THE TYPE STRAIN

Although our experiments with the type strain confirmed the results of those investigators who reported that *S. equi* is unable to produce agglutinins, we were able nevertheless to protect mice with the non-agglutinating serum. For the production of protective serums 4 rabbits were treated with serum broth culture of strain 725 killed at 56°C. for one hour. The doses were given every three or four days, increasing from 0.5 cc. in the first three treatments, to 3 cc. in the last three treatments. All four rabbits received a total of about 40 cc. of killed antigen, then 0.01 cc. of living culture given five days after the preceding dose, followed by 0.1 cc. of living culture two days later. A rise in temperature lasting several days followed the inoculations with living cultures. Four or five days after the temperature returned to normal each rabbit was bled from the heart. No agglutinins could be demonstrated in any of these serums.

TABLE 2

Protocol showing protective action in mice of the serum from rabbit 245 immunized with S. equi, no. 725

The test organism was the homologous strain

CULTURE DILUTION	NORMAL SERUM (CONTROL MICE)	IMMUNE SERUM
10^{-1}		Died* 5 hours
10^{-2}		Died* 20 hours
10^{-3}		Survived
10^{-4}	Died* 27 hours†	Survived
10^{-5}	Survived	Survived
10^{-6}	Survived	Survived

* A pure culture of streptococcus was obtained from the heart blood.

† The minimal lethal dose of Strain 725 is larger than was reported elsewhere in this paper because in this experiment the stock strain was used.

For the protection test white mice were injected intraperitone-

ally with 0.5 cc. of serum, and 24 hours later they were inoculated intraperitoneally with 0.5 cc. of suitably diluted culture. The serum from 2 of the rabbits protected mice against about 10 minimal lethal doses of the homologous strain; the serum from the other two animals gave only slight evidence of protective action. The protocol of the test for one of the serums is given in table 2. The two serums which showed protective action against the homologous strain were pooled, and the pooled serum was shown to protect mice in cross immunity experiments to be reported in another communication.

OTHER S. EQUI STRAINS

Ten strains agreed with the type strain in the differential characters and in every other character studied. (Virulence for mice was determined for two of them, and lysis of human fibrin was determined for four of them.) The strains agreeing with the type strain were nos. 726, 727, 930, 939, 940, 941, 942, 944, 1237 and 1238.

The remaining nine strains included in this study differed from the type strain in one or more differential characters, but were more closely related to *S. equi* than to any other species. They are regarded as atypical *S. equi*.

Two strains, nos. 1225 and 1226, from the same epidemic of strangles, agreed with each other in differential characters, but differed from the type strain in their lack of sensitivity to nascent phage C/594 and B/563 filtrate, and in their ability to grow moderately well in broth. One of these strains was tested for lysis of human fibrin and virulence for mice. It was found to agree with the type strain in those characters.

Seven strains, nos. 865–871, all from mid-western States, differed from the type strain in their lack of sensitivity to nascent phage D/693; and two of them, nos. 867 and 869, differed further from the type strain in their lack of sensitivity to nascent C/594 and to B/563 filtrate. Three of these seven atypical strains were tested for lysis of human fibrin. Two (865 and 867) agreed with the type strain in complete lack of fibrinolytic power; one (870) showed a very slight fibrinolytic power. The virulence

for mice of this group of atypical strains was found to be less than that of the typical strains. A mouse inoculated with 0.02 cc. of culture 865 survived; the minimal lethal dose of the passage strain 867 was 0.0000001 cc.; that of the passage strain 870 was 0.000001 cc. The superiority of the atypical strain 865 over the type strain as an antigen for the production of agglutinins has already been discussed.

The differential characters of the type strain, and of one strain representing each of the variant groups are summarized in table 3. It is to be noted that the variants all agree with the type strain

TABLE 3

The differential characters of typical S. equi and variant groups

The characters of *S. pyogenes* are included for comparison

STRAIN	NUMBER OF STRAINS REPRESENTED	SPECIFIC NAME	SENSITIVITY TO NASCENT PHAGE				SENSITIVITY TO B/563 FILTRATE	FERMENTATION OF		
			B/563	C/594	C/646	D/603		Lactose	Trehalose	Sorbitol
1168		*S. pyogenes*	+	+	+	−	−	+	+	−
725	11	*S. equi*	+	+	+	+	+	−	−	−
1125	2	Atypical *S. equi*	+	−	+	+	−	−	−	−
865	5	Atypical *S. equi*	+	+	+	−	+	−	−	−
867	2	Atypical *S. equi*	+	−	+	−	−	−	−	−

in fermentation reactions, but that there is more or less variation in the phagological reactions. The differential characters of the type strain of *S. pyogenes* are included in the table for comparison.

THE PRECIPITIN GROUPING OF S. EQUI

Four of the typical *S. equi* strains have been studied as to precipitin grouping by other investigators. Lancefield found that strains 725 and 939 belong to her serological group C. Edwards, using Lancefield's technique, found that strains 726 and 727 belong to group C. It remained to be determined whether the atypical strains which are not sensitive to phage D would also belong to the same serological group. Dr. Lancefield very kindly examined the atypical strain 865, and found that it belongs to her group C, as was to be expected.

HUMAN STRAINS REPORTED AS S. EQUI

According to Holman's classification of hemolytic streptococci, fermentation of salicin and failure to ferment lactose differentiate *S. equi* from other hemolytic streptococci. On this basis certain strains from human sources have been reported to be *S. equi*. Fisher studied 9 strains of hemolytic streptococci obtained from the blood of patients with hemorrhagic smallpox during the Detroit epidemic in 1924. He found that 8 of them failed to ferment lactose. Coburn and Pauli reported that 7 out of 44 strains of hemolytic streptococci from rheumatic patients agreed with *S. equi* in failing to ferment lactose. Plummer reported that 7 of the 328 human strains of her collection failed to ferment lactose and therefore belonged to the *equi* group. The majority of strains of the "minute hemolytic streptococci" of Long and Bliss failed to ferment lactose and sorbitol, but fermented trehalose; a few failed to ferment any of the three substances.

Representative strains from the four mentioned collections of human strains of non-lactose fermenting streptococci were examined for characters indicating a relationship with *S. equi*. All of these non-lactose fermenting strains fell into well defined groups, with general agreement between all the strains from each of the four sources. Fisher's strains all differed from *S. equi* in that they fermented trehalose and lysed human fibrin. The strains of Coburn and Pauli resembled Plummer's strains; they were unlike *S. equi* in their lack of sensitivity to nascent P/693 phage; in their ability to ferment trehalose, and in their ability to lyse human fibrin. The strains of Long and Bliss varied among themselves, and yet agreed in certain characters which distinguish this group from *S. equi* and all other hemolytic streptococci.

The above observations on non-lactose-fermenting strains from human sources demonstrate that the single character of inability to ferment lactose does not distinguish *S. equi*. Likewise the inability to ferment all three substances, lactose, trehalose and sorbitol, fails to identify *S. equi*. There are in our collection a number of strains which agree with *S. equi* in their inability to ferment lactose, trehalose and sorbitol but which are otherwise very unlike *S. equi*.

DISCUSSION

In our collection of about 600 strains of hemolytic streptococci, which includes about 150 strains from animal disease sources and about 400 strains from human disease sources, there was no strain agreeing with *S. equi* from any disease other than strangles. This suggests a remarkable disease specificity for *S. equi*, in the variants as well as in the typical strains, with horses as the only host species. The high virulence for experimentally inoculated mice suggests the possibility, however, that mice might be susceptible to spontaneous infection.

The results of this study offer a logical explanation for the inconsistent results of those investigators who have studied the characters of the streptococcus of strangles. It has been a common practice to designate as *S. equi* any streptococcus isolated from a disease in horses diagnosed as strangles. The results of our studies show, however, that more than one species of streptococcus is capable of causing a disease in horses which, correctly or not, is diagnosed as strangles. In our collection there are in addition to the twenty strains of *S. equi* included in the present study, ten other strains with histories of having been isolated from strangles, which, nevertheless, differ from *S. equi* so widely as to appear to deserve to be regarded as separate species. In one group there are four strangles strains which differ from *S. equi* in fermenting trehalose. Strains of this group were isolated from cases of disease in man and in chickens. In another group are 6 strangles strains which differ from *S. equi* in fermenting lactose; and 5 of them are further distinguished from *S. equi* in their ability to ferment sorbitol. Strains of this group were isolated from cases of disease in many species of domestic animals.[1] These two groups of streptococci which include strains from strangles will be described in detail in subsequent papers of this series.

The fact that any strain regarded as the causal organism in a case of strangles has been generally considered to be *S. equi* offers

[1] In a preliminary paper (1935) the group of lactose fermenting strains from strangles was considered as atypical *S. equi*. After further study, however, the group appears to be well enough defined to regard it as a separate species.

an explanation of the controversy as to whether *S. equi* may, or may not ferment lactose. From the present study it may be concluded that *S. equi* does not ferment lactose (see table 3) but that other species of streptococci which are capable of fermenting lactose may produce a disease in horses which is diagnosed as strangles.

Likewise the present study offers an explanation for the discordant results of those investigators who have reported on the pathogenicity of *S. equi*. Our results agree with those of the majority who found that it is highly virulent for mice, but slightly virulent for rabbits and guinea pigs. It appears that those who reported that the streptococcus of strangles is virulent for animals other than horses and mice, must have been working with streptococci which belonged to other species, which, according to our data, may cause spontaneous disease in a variety of other hosts, as well as a disease in horses which is diagnosed as strangles.

There can be no doubt that the group for which strain 725 has been chosen as the type should receive the name *Streptococcus equi*. The strains of this group agree with Schütz's original description in growing poorly or not at all in ordinary media; the type strain was found to be incapable of producing significant agglutinins in rabbits, thus agreeing with the many quoted observations; in their failure to ferment lactose, trehalose and sorbitol, the strains of the group described as *S. equi* agree with the characters of *S. equi* as described by Holth; Adsersen; Ogura; and Edwards.

SUMMARY

A review is given of the literature on *Streptococcus equi*, and the characters of the species are described, as observed in twenty cultures isolated in various parts of the United States and in Denmark (4 strains).

Eleven strains agreed in every character. One of them, no. 725, was chosen as the type strain. Nine strains are regarded as variants, because they differ from the type strain in one or

more characters, and yet resemble *S. equi* more closely than any other species.

In a collection of about 400 strains from human disease sources, not one was classified as *S. equi*. The human strains which had been reported by other investigators to be *S. equi* were found to belong to other groups.

REFERENCES

ADSERSEN, VALD. 1915 Die Spezifizität des Drusestreptococcus, mit besonderer Berücksichtigung des Vergärungsvermögens gegenüber Kohlhydraten u. s. w. Centralbl. f. Bakt. Orig., **76**, 111–117.

ARONSON, HANS. 1903 Weitere Untersuchungen über Streptokokken. Deutsch. Med. Woch., **29**, 439–442.

BROCQ-ROUSSEU, FORGEOT, AND URBAIN, ACH. 1925 Le Streptocoque gourmeux. Rev. Path. Comp. et d'Hyg. Gen., **25**, 257–274; 301–318; 379 397; 451–468; 485–509; 539–546.

COBURN, ALVIN F., AND PAULI, RUTH H. 1932 Observations on the biological characters of Streptococcus hemolyticus associated with rheumatic disease. Jour. Exper. Med., **56**, 633–650.

EDWARDS, PHILIP R. 1933 Further studies on the differentiation of human and animal strains of hemolytic streptococci. Jour. Bact., **25**, 527–536.

EDWARDS, PHILIP R. 1934 The differentiation of hemolytic streptococci of human and animal origin by group precipitin tests. Jour. Bact., **27**, 527–534.

EVANS, ALICE C. 1936 Studies on hemolytic streptococci. I. Methods of classification. Jour. Bact., **31**, 423–437. II. Streptococcus pyogenes. Jour. Bact., **31**, 611–624.

EVANS, ALICE C. 1935 Sensitivity to nascent phage as a character for the differentiation of the beta type of streptococci. Jour. Bact., **29**, 40.

FISHER, L. W. 1933 Studies on hemolytic streptococci isolated from hemorrhagic smallpox. Jour. Lab. and Clin. Med., **19**, 280–295.

GEWENIGER. 1932 Zur Frage der differenzierung des *Streptococcus equi*. Centralbl. f. Bakt., Orig., **124**, 236–238.

HAUPT, H. 1931 Zur Biologie des *Streptococcus equi* und des *Streptococcus agalactiae*. Centralbl. f. Bakt., Orig., **120**, 291–304.

HOLMAN, W. L. 1916 The classification of streptococci. Jour. Med. Res., **34**, 377–443.

HOLTH. 1911 Cited by Jensen in Handb. d. Serumtherapie und Serumdiagnostik. i. d. Vet. Med. Klimmer u. Wolff-Eisner, Leipzig, p. 226.

JONES, F. S. 1919 The streptococci of equines. Jour. Exper. Med., **30**, 159–178.

KOCH, JOS., AND POKSCHISCHEWSKY, N. 1913 Über die Artverschiedenheit des *Streptococcus longus* seu erysipelatos und des *Streptococcus equi* (Druse-Streptococcus). Zeitschr. f. Hyg., **74**, 1–28.

LANCEFIELD, REBECCA C. 1933 A serological differentiation of human and other groups of hemolytic streptococci. Jour. Exper. Med., **57**, 571–595.

LONG, PERRIN H., AND BLISS, ELEANOR A. 1934 Studies upon minute hemolytic Streptococci. I. The isolation and cultural characteristics of minute beta hemolytic streptococci. Jour. Exper. Med., **60**, 619–631.

MAASS. 1913 Zur Frage der Streptokokken-Differenzierung durch kohlehydrathaltige Nährboden. Centralbl. f. Bakt., Abt. I, Ref. **57**, Beiheft, 258–262.

OGURA, KISAJIRO. 1929 Ueber Drusestreptococcus, mit besonderer Berücksichtigung seiner Spezifität. Jour. Jap. Soc. Vet. Sci., **8**, 174–203.

PIORKOWSKI, G. 1902 Ueber Streptokokkensera. Berl. Klin. Woch., 1125.

PLUMMER, HELEN. 1934 The fermentation of sorbitol and trehalose by haemolytic streptococci from various sources. Jour. Bact., **27**, 465–472.

POELS, J. 1888 Die Mikrokokken der Druse der Pferde (Coryza contigiosa equorum). Fortschritte der Medicin, **6**, 4–7.

PRICOLO, ANTONIO. 1910 Recherches expérimentales sur le streptocoque de la gourme. Centralbl. f. Bakt., I Abt., Orig., **55**, 352–360.

SAND, G., AND JENSEN, C. O. 1888 Die Aetiologie der Druse. Deutsche Zeitschr. f. Thiermed., **13**, 437–464.

SANDE, V. 1927 Die Veränderung der Lakmusmilch durch Streptokokken. Dtsch. tierärztl Wschr., **35**, 329–331.

SCHNÜRER, JOSEF. 1903 Untersuchungen über die Immunität bei der Druse Zeits. f. Thiermed., **7**, 286–307.

SCHÜTZ. 1887 In Friedberger and Fröhner's Lehrbuch der Speciellen Pathologie und Therapie der Hausthiere. Stuttgart, **2**, 351.

SCHÜTZ. 1888 Der Streptococcus der Druse der Pferde. Archiv f. Thierheilkunde, **14**, 172–218.

STOLPE, BERNHARD. 1906 Ueber die mittelst Agglutination nachweisbaren Beziehungen des Streptococcus equi zu den vom Menschen stammenden Streptokokken. Fortschritte der Vet. Hyg., 265–286.

STRAATEN, HENDRIK VAN. 1912 Die Spezifität der Drusestreptokokken. Inaug. Dessert., Berne.

TODD, A. G. 1910 Strangles. Jour. Comp. Path. and Ther., **23**, 212–229.

THE SPONTANEOUS TRANSFORMATION OF PNEUMOCOCCUS TYPE V TO TYPE II[1,2]

L. A. BARNES AND ELEANOR C. WIGHT

Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health, Boston, Massachusetts

Received for publication, July 3, 1936

A search of the literature has failed to produce definite evidence that a virulent pneumococcus may undergo spontaneous conversion from one type to another, although, in view of some of the data to be presented, one is led to suspect that such phenomena may have occurred but have gone unrecognized. That the question has been considered, is indicated by the work of Megrail and Ecker (1924) who reported experiments which led them to conclude that the pneumococcus remains type-stable under conditions which cause a variability in agglutination of typhoid bacilli and other organisms. The observations of various investigators such as Alloway (1932), Dawson (1930), and others, have shown, however, that a pneumococcus with the specific characteristics of a given type, may be transformed into a different type by first depriving the organism of its type-specificity and then subjecting it to an environment, either *in vitro* or *in vivo*, containing substances obtained from a culture of the type into which it is desired to convert the degraded organism.

[1] This work was carried out in part as one of a series of studies on pneumonia conducted under a grant from the Commonwealth Fund of New York.

[2] Our experience has been duplicated in part by that of Dr. Henry Welch of the Connecticut State Department of Health. In July, 1934 he obtained a type V pneumococcus culture from Miss Cooper. This culture was used in the preparation of serum for the Neufeld test and was found to be satisfactory at first. The culture was carried on sheep-blood agar until November, 1935 when it was passed through mice and upon Neufeld testing was found to be a type II. Careful examination of stock cultures showed no apparent confusion of strains and the transformed type V culture was discarded. Dr. Welch, in a discussion of these results, expressed the belief that a conversion of type V to type II may have occurred in a manner corresponding to that outlined in this paper.

This report deals with evidence which indicates that a virulent pneumococcus type V may, under certain conditions, change quite abruptly into a type II. The fact that type II strains were divided into subgroups by Avery (1915) and later by Stillman (1919), and the subsequent classification by Cooper and her associates (1929) of the Avery Subgroup II A as type V, forms a basis for speculation as to the chemical, or other, bases for type differentiation.

EXPERIMENTAL DATA

On November 9, 1931 a culture of type V pneumococcus was received from Miss Georgia M. Cooper of the New York City Department of Health. On November 20, 1931 this culture showed a 3+ agglutination[3] in a final dilution of 1:40 of the homologous serum also supplied by her. There was only a slight cross-reaction with type II serum (1:2 dilution). Eighteen and a half months later the agglutination titer with type V serum had decreased so that only a 1+ reaction was obtained in a 1:5 serum dilution, although there were traces of agglutination in higher dilutions. After another ten months the agglutination reactions were 1+ in 1:2 and ± in 1:5 serum dilutions. The possibility that the culture had undergone a type transformation was overlooked and the culture was discarded.

A new type V culture (Pn V-C) was obtained May 2, 1934 from Miss Cooper, and, with the same lot of serum as before, was found to exhibit satisfactory type-specific agglutination. On November 1, 1935, however, it was noticed that the agglutination with type V serum was again growing weaker (2+ in 1:5 dilutions and only traces in higher dilutions). A culture made from the heart's blood of a mouse was tested the following day and the agglutination reactions showed that the culture which was formerly a type V had become predominantly a type II as shown in table 1 (Culture November 1).

These findings suggested that the culture had undergone an actual transformation of type; and the experiments to be described were devised to determine the validity of this hypothesis.

[3] All agglutination results are after two hours incubation in a water bath at 41°C. Storage overnight produced no essential changes.

To appreciate the conditions under which this change took place a brief description will be given of the care of the pneumococcus stock culture collection. When a new culture is obtained it is transferred to pneumococcus broth and a routine mouse passage made as soon as convenient. Using the same broth culture, the usual identity tests (Gram stains, bile solubility, inulin and milk

TABLE 1

Agglutination tests on transplants of mouse passage cultures

CULTURES DATES OF MOUSE PASSAGE	SERUMS	FINAL DILUTIONS OF SERUMS					
		1:5	1:10	1:20	1:40	1:80	1:160
November 1*	†V H	2	0	0	0	0	0
	V R	0	0	0	0	0	0
	II H	4	4	3	1	0	0
October 25	V H	1	0	0	0	0	0
	V R	0	0	0	0	0	0
	II H	4	4	3	0	0	0
October 18	V H	1	1	2	2	2	1
	V R	1	3	3	3	3	0
	II H	3	3	3	0	0	0
October 8	V H	2	3	3	3	1	0
	V R	3	4	4	3	3	0
	II H	1	1	1	±	0	0
Control. Known pure, type II	V H	2	0	0	0	0	0
	V R	0	0	0	0	0	0
	II H	4	4	4	1	0	0

*October, November, dates are all 1935.
†V H = Type V horse serum.
V R = Type V rabbit serum.
II H = Type II horse serum.

fermentation, and agglutination tests) are performed. All stock cultures are passed through mice at least once each week, some twice a week, and those used routinely for animal immunization five times a week. One or two fresh broth cultures each week are kept and the older ones discarded. Individual labeled glass jars are used for mice injected with each culture and the jars are washed each time after use. Syringes are boiled thoroughly

before and after use and rinsed with alcohol and ether. Once each month the cultures are all subjected to routine identification tests and at least two blood-agar slants made from heart's blood of mice injected with the respective cultures. In addition, sealed glass capillary pipettes of heart's blood are prepared at indefinite intervals. The cultures are stored at about 4°C.

Attempt to determine approximate time of transformation. Since two different type V cultures had been observed to lose their type-specific agglutinability under similar conditions, and evidence was obtained (table 1) that this was due to a transformation of type, some of the series of routine mouse passage cultures were transferred to broth. Agglutination tests performed on four of these cultures showed that the change was in progress on October 18, 1935 and was essentially complete on November 1, 1935 (table 1).

To demonstrate further that the culture of October 18, 1935 actually contained a mixture of type V and type II pneumococci, three steps were taken as follows: (*a*) A 1:1,000,000 dilution in peptone water was streaked on a blood-agar plate. Fifteen well-isolated colonies proved to be type V and five were type II, thus indicating a mixture of approximately 75 per cent type V and 25 per cent type II organisms in the culture. (*b*) One mouse was injected intraperitoneally with a mixture of equal parts of a 1:1000 dilution of the culture and undiluted type V rabbit serum, while a second was injected with the culture dilution plus type II rabbit serum. After twenty-four hours, the mice were autopsied and cultures made of the heart's blood of each. Agglutination tests indicated that this procedure had resulted in a complete separation of the two types of pneumococci present in the original culture. This method was suggested by the work of Griffith (1928). (*c*) Using type II and type V rabbit serums an agglutination test was performed, and the 1:40 dilutions centrifuged lightly. One-half cubic centimeter doses of the supernatants were injected into mice and autopsy cultures typed. Here again it was found that a separation had been effected.

To demonstrate that the culture had originally been pure type V and eventually became pure type II, a broth culture was made

from a blood agar slant from a mouse passage of September 4, 1935, and a second broth culture transfer from a mouse passage culture of November 8, 1935. Blood agar streak plates were made of each from a 1:1,000,000 dilution and all of twenty-three well-isolated colonies from the September 9 culture proved to be pure type V cultures, while all twenty-three of those from the November 8 culture were pure type II pneumococci as indicated by agglutination with monovalent horse serums.

Attempts were made to effect a transformation, under controlled experimental conditions, of several of the type V cultures by injecting mice subcutaneously with minute amounts of the cultures together with relatively large amounts of heat-killed virulent type II pneumococcus vaccine, a modification of Griffith's technic (1928). All such attempts failed, presumably because of the virulence of the type V cultures, and further trials of this nature were not made.

Final attempt to effect transformation under experimental conditions. Due to the fact that the spontaneous conversion of type V to type II occurred during the course of routine mouse passages (one or two each week) it was decided to determine whether frequent serial mouse passages would bring about the change in a pure type V culture which had been made shortly prior to the transformation. A mouse passage culture made October 4, 1935 was selected and transferred to broth December 23, 1935 to serve as the original culture for this experiment. Mouse passage cultures were made on December 28 and 31, 1935, and January 2, 3, 6, 7 and 8, 1936. The results of agglutination tests performed with these cultures show that the transformation from a type V to a type II pneumococcus occurred quite abruptly, December 31, 1935 to January 2, 1936 (table 2). Essentially the same results were obtained with rabbit serums except that the January 2, 3 and 6, 1936 cultures still showed some type V agglutination which was lacking in the January 7 and 8, 1936 cultures. The December 23, 28 and 31, 1935 cultures were completely negative for type II organisms.

Since the change from type V to type II occurred during the period between December 31, 1935 and January 2, 1936, further

mouse passage of the December 31 culture was tried to determine whether a single passage would again effect the conversion and, if so, under what conditions. Six mice were injected intraperitoneally with 0.5 cc. of an eighteen-hour transfer of the December 31 culture and placed in thoroughly disinfected jars. Cultures of the peritoneal exudates of three of the mice were made at one- and four-hour intervals after injection. All six mice were chloroformed after six hours and cultures made from both peritoneal exudate and heart's blood of each mouse. The

TABLE 2

Agglutination tests on transplants of serial mouse passage cultures of Pn 5-c October 4, 1935

TYPE II HORSE SERUM FINAL DILUTIONS						CULTURES*	TYPE V HORSE SERUM FINAL DILUTIONS					
1:5	1:10	1:20	1:40	1:80	1:160		1:5	1:10	1:20	1:40	1:80	1:160
0	0	0	0	0	0	December 23†	3	3	4	3	3	0
0	0	0	0	0	0	December 28	3	3	3	3	2	1
0	0	0	0	0	0	December 31	3	3	3	3	3	2
4	4	3	±	0	0	January 2	0	0	0	0	0	0
4	4	4	1	0	0	January 3	0	0	0	0	0	0
4	4	3	0	0	0	January 6	0	0	0	0	0	0
4	4	3	0	0	0	January 7	0	0	0	0	0	0
4	4	4	0	0	0	January 8‡	0	0	0	0	0	0
0	0	0	0	0	0	Known pure, type V	3	3	3	3	3	0
4	4	3	0	0	0	Known pure, type II	0	0	0	0	0	0

* Transplants made January 8, 1936.

† December dates 1935. January dates 1936.

‡ Direct culture from heart's blood of mouse.

results of agglutination tests made on these cultures with type II and type V horse serums show that even after one hour in the peritoneal cavity of mice, type II organisms could be easily demonstrated. In only one mouse did the heart's blood show pure type II. This is significant because in the peritoneal exudate of this mouse at the same time both type II and type V organisms were well represented.

The mixed type II and type V heart's blood culture of one of these mice was injected into a normal mouse from which a

heart's blood culture was taken after six hours and typed. This additional mouse passage completed the change from type V to type II pneumococcus.

Due to the fact that the October 18, 1935 culture agglutinated with both type II and type V serums (table 1) and agglutination tests on single colony cultures indicated a mixture of approximately 75 per cent type V and 25 per cent type II organisms, it seemed pertinent to perform agglutination tests and mouse passages with measured mixtures of the two types known to be pure. Using eighteen-hour broth cultures of type II and type V organisms, mixtures were made in the proportions of 1:99, 5:95 and 10:90 respectively and agglutination tests carried out. The results indicate that such mixtures may contain at least 5 per cent of type II organisms which are undetectable by the agglutination test with horse serum. The presence of type II organisms in mixtures containing 10 per cent or more of them is likely to be detected.

One-half cubic centimeter of a mixture containing 1 part of type II and 9999 parts of type V pneumococci was injected intraperitoneally into a mouse. Cultures were made from the peritoneal exudate and heart's blood when the mouse was chloroformed six hours later. The heart's blood culture was injected into a second mouse and a heart's blood culture made as before. Agglutination tests on these cultures show that a mixture containing this relatively minute number of type II organisms and a large number of type V pneumococci will, after serial mouse passage, show no type V pneumococci in the heart's blood culture. These findings offer an explanation for the change of the December 31, 1935 culture from an apparent type V to type II following mouse passage, since there were at least some type II organisms in this culture, as will be shown later; they do not, however, account for the presence of the type II pneumococci in the original culture supposedly pure type V.

As a matter of interest, it may be mentioned that the use of antipneumococcic-serum-agar for the identification of colonies, as recommended by Sia and Chung (1932), was tried with mixtures of type II and type V pneumococci with very encouraging

results. Sufficient data were not obtained, however, to warrant detailed discussion, nor was this method tried until after the experiments on transformation of type had been concluded.

Later experiments. The experiments were interrupted for about a month during which no transfers or mouse passages were made of the cultures dated December 23, 28 and 31, 1935 and January 2, 3, 6, 7 and 8, 1936. This interval may have made some of the later experiments less reliable for there is reason to believe that type II organisms may die out more rapidly than type V when stored in culture media. Therefore we are not inclined to attach much importance to the second of the following experiments. Transfers of the eight cultures were made February 9, 1936, and all were recovered except that dated January 8, 1936. Agglutination tests with these cultures produced surprising results; the January 2 culture appeared to be pure type V and the January 3 culture pure type II, whereas, when tested before, the transformation had occurred between December 31, 1935 and January 2, 1936. In view of these findings, agglutination tests were performed on cultures of twenty-four single colonies isolated on blood agar from eighteen-hour growths of each of the December 31, 1935, January 2 and 3, 1936 cultures. The results were as follows: December 31, 1935 culture, twenty-three colonies type V, one colony type II; January 2, 1936 culture, twenty-four colonies type V, none type II; January 3, 1936 culture, twenty-three colonies type II, one colony type V. Although the question of chance sampling cannot be ignored, these results indicate that a transformation of type occurred between December 31, 1935 and January 3, 1936.

Attempts were made to obtain a transformation of the January 2, 1936 culture from type V to type II by serial mouse passages. Over thirty such passages were made without evidence of conversion of type and the procedure was discontinued.

DISCUSSION

The data presented in this report do not prove, but do suggest very strongly, that under certain routine conditions a type V pneumococcus will become spontaneously converted into a type

II. The factor or factors responsible for this instability of type are obscure. The experimental work described suggests methods by which such conversion of type may be demonstrated, and how it may be prevented. If one accepts the hypothesis that an actual transformation of type has been observed, there are certain practical applications which should be considered.

In the routine preparation of serums for diagnostic use, especial care may be required with known closely related types of pneumococci. With the developing interest in the use of therapeutic serums for type V and type VIII, for example, it will be of economic importance, both from the clinical and manufacturing standpoints to have available strictly specific typing serums. Use of the Griffith technic, or of Sia and Chung's method, for the purpose of maintaining type-specific stock cultures may be found desirable.

From the standpoint of the clinician it would seem of importance to make repeated typings on pneumonia cases in which the original diagnosis was, for example, type V. It is not impossible that, in some instances, a diagnosed type V case might later become type II and respond to treatment with that serum. Possibly in such cases, a bivalent type V and type II serum would be more effective than either type alone.

From the theoretical standpoint, one is tempted to speculate upon the chemical, or other, bases of type-specificity. Although the type V pneumococcus, as classified by Cooper, was formerly designated Subgroup II A by Avery, there is little question but that it is a separate type. On the other hand, the evidence indicates that the relationship between the two types is very close. It seems possible that type V may differ only slightly from type II in the structure of the capsular polysaccharide. The preparation and analysis of type V SSS would aid in settling this question. Such procedures might profitably be extended to other closely related types.

Of interest also is the question of antagonism or selection in a mixed culture containing type V and type II organisms. Some evidence was obtained which suggested that upon storage without transfer, type II organisms would disappear, possibly because of

more rapid autolysis, leaving only type V. Obviously, the question of chance sampling cannot be ignored in such a case. Definite experimental evidence was obtained that, upon injecting mice intraperitoneally with mixtures of the two types, even when relatively minute numbers of type II were present, the type II exhibited a tendency to invade the bloodstream first, so that pure type II cultures could be recovered from the heart's blood. Explanation of this observation must await further research.

SUMMARY

Observations are recorded which lead to the belief that a pure type V pneumococcus may be transformed spontaneously into a type II. Methods of detecting and preventing such conversion are presented. Certain practical and theoretical considerations of such a phenomenon are discussed.

REFERENCES

ALLOWAY, J. L. 1932 Jour. Exper. Med., **55**, 91.
AVERY, O. T. 1915 Jour. Exper. Med., **22**, 804.
COOPER, G., EDWARDS, M., AND ROSENSTEIN, C. 1929 Jour. Exper. Med., **49**, 461.
DAWSON, M. H. 1930 Jour. Exper. Med., **51**, 123.
GRIFFITH, F. 1928 Jour. Hyg., **27**, 113.
MEGRAIL, E., AND ECKER, E. E. 1924 Jour. Infect. Dis., **35**, 1.
SIA, R. H. P., AND CHUNG, S. F. 1932 Proc. Soc. Exp. Biol. and Med., **29**, 792.
STILLMAN, E. G. 1919 Jour. Exper. Med., **29**, 251.

BACTERIAL GROWTH AT CONSTANT pH

APPARENT OXIDATION-REDUCTION POTENTIAL, ACID PRODUCTION, AND POPULATION STUDIES OF LACTOBACILLUS ACIDOPHILUS UNDER ANAEROBIC CONDITIONS

LEWIS G. LONGSWORTH AND D. A. MACINNES

Laboratories of The Rockefeller Institute for Medical Research

Received for publication July 6, 1936

In the two earlier articles by the authors (1935, 1936) investigations, on the growth of *Lactobacillus acidophilus*, were described in which the pH of the medium was kept constant at any desired value by means of an automatic regulator, and in addition the temperature and the composition of the gas mixture saturating the medium were controlled. The progress of the growth was followed in the later experiments by measurements of the acid production, apparent oxidation-reduction potentials (E_h values), and the bacterial population. Some interesting inter-relations of these measurements were observed for which the second paper of this series may be consulted. In particular a connection was found between the E_h value and the *rate* of acid production. The E_h values were, however, found to be much affected by the presence of oxygen in the saturating gas.

It was considered, therefore, desirable to continue the investigations to include studies of bacterial growth under anaerobic conditions. However since it has been shown that carbon dioxide is essential for such growth, it was necessary to prepare an inert gas (nitrogen) free from oxygen but containing carbon dioxide. The method for obtaining such mixtures will first be described, together with an outline of changes made in the design of the culture flask. This will be followed by a discussion of the results of experiments, on the growth of *L. acidophilus* under

anaerobic conditions with automatic control at pH 6.0 during which measurements of oxidation-reduction potentials, acid formation, and population were carried out.

EXPERIMENTAL ARRANGEMENTS FOR ANAEROBIC GROWTH

The method used for preparing oxygen-free mixtures of nitrogen and carbon dioxide depends upon the decomposition of solid

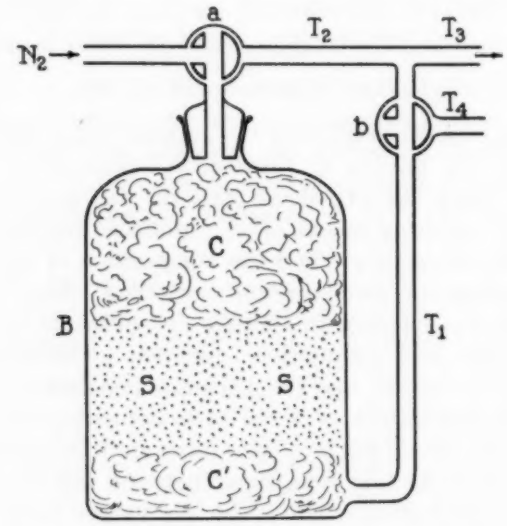


FIG. 1. APPARATUS USED IN THE PREPARATION OF OXYGEN-FREE MIXTURES OF NITROGEN AND CARBON DIOXIDE

sodium bicarbonate at moderately high temperatures according to the equation

$$2\ NaHCO_3 = Na_2CO_3 + H_2O + CO_2 \qquad (I)$$

The tank nitrogen is first passed through a quartz tube containing finely divided copper at 500°C. to remove the 0.2 per cent of oxygen present as impurity. The gas then passes into the top of a two-liter Pyrex bottle, shown in figure 1, where it is spread by the cotton plug *C* before passing through the layer of sodium

bicarbonate, S, and finally passes through another cotton plug C' before leaving through the tube T_1–T_3. The gas then flows to the culture flask as described in previous papers.

On its passage through the bicarbonate the nitrogen picks up carbon dioxide and water vapour, the latter being subsequently condensed out of the mixture. With a gas stream of 3 liters per hour and the bicarbonate at a temperature of 79.8°C., maintained by use of an oil thermostat, the resulting gas mixture contained 3 per cent of carbon dioxide. This percentage depends upon the rate of flow since it is considerably below the equilibrium value for reaction I at this temperature, as determined by Caven and Sand (1911). Experiment showed, however, that the composition of the issuing gas is quite constant over long intervals of time if the temperature and rate of flow are held constant. Precaution must be taken to prevent the moisture from condensing in the bottle and "caking" the solid reagents. By manipulation of the stopcocks a and b of figure 1, it is possible to by-pass the stream of nitrogen through the tube T_2–T_3 and at the same time relieve accumulated pressure in the bottle B through the tube T_4. This arrangement was used in a test, to be described later, of the growth of the organism with and without carbon dioxide.

The oxygen-free mixtures of carbon dioxide and nitrogen pass into the modified[1] culture flask shown in figure 2 through a sterile cotton plug in the tube O, enter the medium at the point T, and escape through a cotton plug inserted in tube A. In our early measurements of E_h, using the flask described in the first paper of this series and gas mixtures containing about 0.2 per cent of oxygen, it was observed that these potentials frequently rose a few millivolts for a short interval after each withdrawal of

[1] It may be observed, by comparison of figure 2 of this paper with the corresponding figure of our first paper (1935), that three ground glass joints have been eliminated in the later model. This has reduced the cost of the apparatus and has increased the ease with which it may be sterilized and manipulated. The conical surface E fits into the apparatus (figure 2 of our second paper (1936)) used for sterilization and insertion of the electrodes, and provision has been made in the hard rubber stopper, R, (figure 2 of the present paper) for separating the glass electrode G from the gold, Au, and platinum, Pt, electrodes so that the metal electrodes may be cleaned with hot nitric acid.

a sample. This was probably due to the atmospheric oxygen which was drawn into the culture flask by the removal of the sample or was able to diffuse into the flask against the counter current of gas escaping through this opening. This contamination with atmospheric oxygen has been eliminated in the experiments reported in this paper by constricting the opening as shown

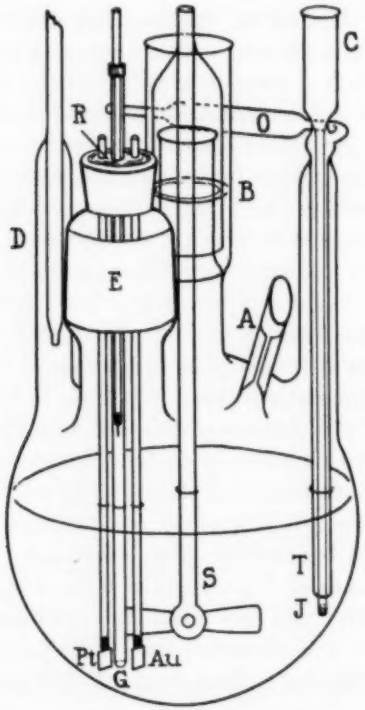


FIG. 2. MODIFIED CULTURE FLASK

at *A*, figure 2, with the result that the velocity of the gas escaping through this outlet is greatly increased. Samples are now taken from the culture flask with the aid of a pipet whose tip is ground to fit a stainless steel needle having a diameter somewhat less than that of the capillary outlet. Moreover, the sampling pipet is filled at a lower rate than that at which gas is being

passed into the flask so that the liquid drawn from the flask is replaced by this gas and not by air drawn in through the opening *A*. The adoption of this technique has resulted in smoother E_h time curves than those obtained in previous work.

Complete absence of the diffusion of atmospheric oxygen through the oil seal *B* provided for the stirrer *S*, figure 2, would be difficult to prove. However, it was observed that by allowing the gas entering at *T* to escape through this oil, thus providing a counter current to such diffusion, no effect was observed on the E_h measurements. Possible contamination with the oxygen dissolved in the solution of alkali that is added at intervals in controlling the pH has been eliminated by de-aeration of this solution with pure nitrogen, after which it was protected from atmospheric oxygen and carbon dioxide with the aid of a nitrogen-filled, collapsible, gas-tight bag.

EFFECT OF CARBON DIOXIDE ON L. ACIDOPHILUS CULTURES

Having taken the precautions described in the preceding section for the anaerobic growth of *L. acidophilus* in the presence of carbon dioxide, it seemed desirable to obtain additional confirmation of the necessity of this gas. Consequently a culture medium, through which pure nitrogen was passing, was inoculated in the usual manner with 0.07 per cent of a 17-hour culture. For 22 hours after inoculation there was no evidence of activity on the part of the culture. By turning stopcocks *a* and *b* of figure 1 an oxygen-free mixture of nitrogen and carbon dioxide (3.5 per cent) was then substituted for the nitrogen, and after an unusually long lag period the culture developed in a normal manner. The lag period, counted from the time of inoculation, was 32 hours, or 10 hours if counted from the time at which carbon dioxide was introduced. Had carbon dioxide been present at the time of inoculation, the lag period would have been only about 1 hour. It is not possible from this experiment alone to decide whether the long lag period following the introduction of carbon dioxide into the culture was due to a loss of viability on the part of the inoculum, or whether lack of carbon dioxide

during the first 22-hour interval induced the transition of the bacteria of the inoculum into a resting phase from which they slowly recovered in its presence.

Depriving a fully established culture of carbon dioxide has no appreciable effect upon the subsequent course of the growth cycle. Thus in one experiment, pure nitrogen was substituted for the nitrogen-carbon dioxide mixture 30 hours after inoculation. Following this alteration of the gas phase the acid production curve falls slightly below those for experiments in which the culture was not deprived of carbon dioxide but the differences are small. Gladstone, Fildes, and Richardson (1935) have emphasized, however, that an actively metabolizing culture may be producing carbon dioxide faster than it can be swept out. The bicarbonate ions which are present in solution following the original equilibration of the medium with the N_2–CO_2 mixtures also act as a reservoir of CO_2.

APPARENT E_h VALUES DURING ANAEROBIC GROWTH

Our early measurements of the apparent oxidation-reduction potentials in bacterial cultures were made with the aid of gold electrodes. It was shown (1936) that two such electrodes agree closely with each other throughout an experiment. Since, however, many E_h measurements (Hewitt (1935)) have been made with platinum electrodes one of the gold electrodes was replaced by platinum, as shown in figure 2, in order to make a direct comparison of the behavior of the two metals.

When placed in the medium as it comes from the sterilizer the gold potential, E_h (Au), is usually about 250 mv. positive, using a value of +246 mv. for the potential of the 3.5 N calomel electrode against the normal hydrogen electrode. The platinum, apparently more responsive to the atmospheric oxygen dissolved in the medium, gives a value of +400 to + 500 mv. During preliminary saturation with the nitrogen-carbon dioxide mixtures E_h (Au) drops to about zero, which appears to be a value characteristic of the de-aerated sterile medium (compare, for example, Coulter (1928–29)). If the equilibration continues long enough, i.e., 2 days, the E_h (Pt) also approaches this value.

TABLE 1

Apparent oxidation-reduction potentials and acid production observed in a typical experiment on the anaerobic growth of L. acidophilus with automatic pH control*

Temperature, 38°. pH, 6.0. Medium: 2 per cent neopeptone; 8 per cent galactose. Gas: CO_2 2.6 per cent; N_2 97.4 per cent. Titrating fluid: 1.94 N NaOH.

1	2	3	4	5
TIME	E_A(Au)	E_A(Pt)	TIME	ACID PRODUCTION A
hours	*millivolts*	*millivolts*	*hours*	*milli-equivalent/liter*
0.50	+7	+209	0.00	0.0
2.00	−1	+181	2.00	0.0
3.50	−11	+151	4.00	0.2
4.35	−25	+130	6.00	0.4
6.70	−75	+65	8.00	0.9
7.70	−121	+26	10.00	1.3
8.75	−149	−31	12.00	3.2
9.75	−162	−80	14.00	6.3
11.00	−171	−112	16.00	12.4
12.55	−188	−142	18.00	21.2
13.75	−188	−152	20.00	34.2
15.75	−192	−163	22.00	52.3
17.85	−198	−171	24.00	72.6
19.85	−198	−178	26.00	98.9
21.80	−194	−175	28.00	133.9
23.75	−189	−178	30.00	171.3
25.75	−180	−180	32.00	210.5
27.75	−173	−189	34.00	247.2
29.80	−165	−195	36.95	294.1
31.95	−159	−199	48.00	425.0
33.75	−155	−204	50.00	443.0
36.90	−159	−209	55.00	477.5
47.75	−171	−209	72.00	559.4
54.75	−180	−210	83.00	587.4
71.75	−191	−207	100.00	608.6
84.75	−188	−203		
99.75	−192	−198		

* The data of this table were obtained in the same experiment as those of table 3 in a paper by Longsworth (1936). The two tables taken together consequently illustrate the results obtained in a typical experiment on the anaerobic growth of *L. acidophilus*.

The chief function of the initial saturation, however, is to establish the equilibrium concentration of bicarbonate ion in the sterile medium, as discussed in our first paper (1935). This requires less time than the stabilization of the E_h, particularly E_h (Pt). Consequently, many of our cultures were inoculated before the E_h (Pt) characteristic of the medium had been attained.

Table 1, which is self-explanatory, gives the E_h values and the acid production, A, obtained during a quite typical experi-

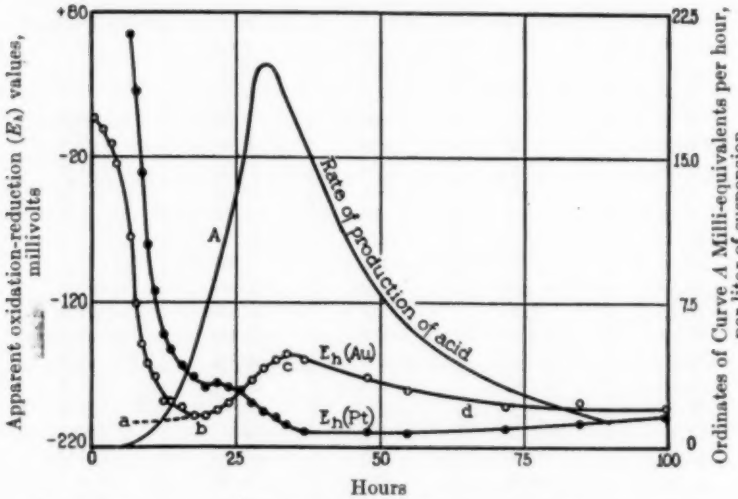


FIG. 3. VARIATION, WITH TIME, OF THE RATE OF ACID PRODUCTION AND THE APPARENT OXIDATION-REDUCTION POTENTIALS

ment. In figure 3 the two sets of E_h values and the rate (dA/dt) of acid production are plotted as functions of the time. It is evident that the E_h (Pt) time curve differs markedly from that obtained with a gold electrode. The reasons for this difference remain obscure but are undoubtedly related to the fact that different metals attain equilibrium at different rates with the electromotively active materials in the solution with which they are in contact (see, for example, Michaelis and Flexner (1928)). If, as in this case, the relative concentrations of such materials

are constantly changing, different potential-time curves will be observed. The trends of both the E_h (Au) and E_h (Pt) time curves, after the E_h (Au) reaches a minimum, were found in a number of experiments to be reproducible within a few millivolts.

It is an interesting fact that the first evidence of activity on the part of a culture after inoculation is a decrease in E_h. In cultures grown with pH control this drop begins several hours before alkali absorption or detectable changes in turbidity (optical density) are observed. As the rate of acid production, curve *A* of figure 3, increases, the E_h (Au) drops rapidly to a minimum of about -190 mv. and then rises, with the rate of acid production, to a maximum of about -150 mv. soon after the maximum in dA/dt. This is followed by a decline of both the E_h (Au) and dA/dt until at 100 hours the E_h (Au) has returned asymptotically to the minimum value of -190 mv. A similar series of changes of potential have been observed by Gillespie and Rettger (1936) in *L. acidophilus* cultures grown without pH control. They ascribe the rise to a maximum and subsequent drop to changes of the pH of the culture. Our cultures, however, were grown at constant pH and the trends of E_h (Au) observed by us cannot, therefore, be ascribed to such changes.

Regardless of the potential of the platinum electrode at the time of inoculation it is more "sluggish" than the gold during the initial downward drift of the E_h. At the time that the E_h (Au) time curve is passing through a minimum there is always evidence of a plateau in the E_h (Pt) time curve but the downward trend is resumed and a minimum is reached somewhat later than the time at which dA/dt is a maximum. It is only in the old culture when the metabolic rate is low and the poising effect of the products of metabolism is high that the E_h (Au) and E_h (Pt) curves approach a common value of about -190 mv.

In any attempt to correlate E_h measurements with the metabolism of the culture one is probably justified in ignoring the initial rapid drop of the E_h since this is due to the replacement of the poising system of small capacity characteristic of the deaerated sterile medium by one of increasing capacity character-

istic of the bacterial metabolism. The course of the E_h (Au) time curve produced by the culture itself is probably represented by a curve through the points *a*, *b*, *c*, *d* of figure 3. The symmetry between this curve and that of the rate of acid production, dA/dt, suggests that the E_h (Au) values are nearer than the E_h (Pt) values to a measure of the instantaneous ratio of electromotively active oxidizing and reducing substances in solution.

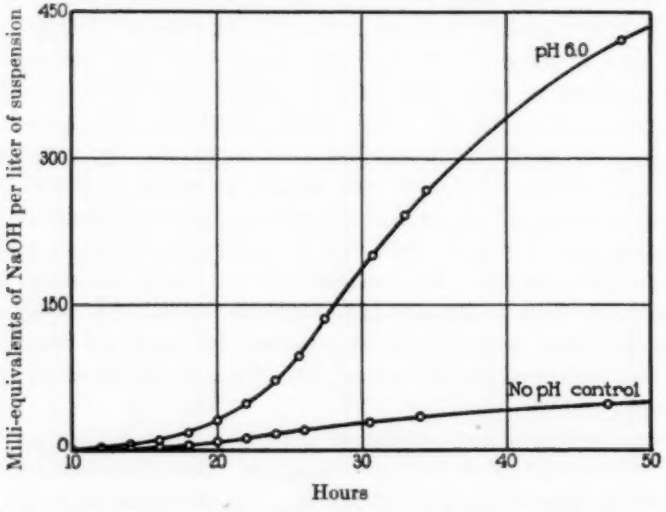


FIG. 4. ACID PRODUCTION OF CULTURES OF L. ACIDOPHILUS GROWN WITH AND WITHOUT pH CONTROL

Medium: 2 per cent neopeptone, 8 per cent glucose

The value of -190 mv., to which E_h (Au) first falls and later, together with E_h (Pt), returns, seems to be a characteristic of the organism at pH 6.0. It has been observed in all of our experiments in which the growth was under approximately anaerobic conditions, in spite of changes in the nature and concentrations of the constituents of the medium. Moreover, the same potential has been observed by Rogers and Whittier (1928) in the formation of lactic acid by *Streptococcus lactis*.

It is not possible, with the experimental data at hand, to

identify the particular system or systems of electromotively active materials responsible for the potentials observed. The only system that appears to be relevant and which has been adequately studied is the lactate-pyruvate system. Barron and Hastings (1934) have observed that

$$E_h = -0.118 - \frac{RT}{2F} \ln \frac{(\text{lactate}^-)}{(\text{pyruvate}^-)}$$

at 35° and pH 6.0. If this system were contributing, reversibly, to the potential of −190 mv. observed in our culture it would indicate an apparently quite reasonable ratio of pyruvate to lactate of about 1 to 100.

The E_h values reported in our second paper (1936) were obtained in experiments in which the gas mixture contained about 0.2 per cent of oxygen. With this oxygen content and the rates of flow used by us, the actively metabolizing culture was apparently able to remove the oxygen (compare, for example, Hegarty (1936)) as fast as it passed into solution. Consequently the minimum E_h observed, −180 mv., was not very different from that attained in anaerobic growth. The presence of this amount of oxygen in the entering gas had the effect, however, of delaying the appearance of the minimum in the E_h time curve until dA/dt is a maximum, as shown in figure 4 of our second paper. Moreover, the final upward drift of the E_h time curve, in contrast with the downward drift of E_h (Au) observed in anaerobic growth, may be due to this trace of oxygen.

If the entering gas contains more oxygen than the culture can reduce, the minimum in the E_h time curve, although occurring soon after the maximum in dA/dt, has a value much more positive than in anaerobic growth. Moreover, this minimum value, unlike that observed under approximately anaerobic conditions, depends upon the value of $\left(\frac{dA}{dt}\right)_{max.}$ Thus, in three experiments in which the gas contained 2 per cent of oxygen the minimum values of E_h were +56, +100, and +159 mv. while the corresponding maximum rates were 8.0, 6.0, and 3.0 milli-equivalents

per hour per liter of suspension. These variations in $\left(\frac{dA}{dt}\right)_{max.}$ were the results of alterations in the composition of the medium.

We may therefore conclude from our determinations of E_A time curves at constant pH that, although much more remains to be done in this field, the trends of such curves are definitely related to the *rates* of acid production of these cultures. This does not exclude the possibility of correlations of such curves with the rates of cell multiplication, as suggested by Clifton, Cleary, and Beard (1934). However, in our work the best correlation is obtained with the acid production.

THE PHYSIOLOGY OF CULTURES GROWN WITH AND WITHOUT pH CONTROL

In the development of an acid-producing culture the amount of acid formed in a small interval of time, Δt, is the product of that interval, the population (plate count) of the culture, C, and the rate of acid production per unit of population, Φ. The last two factors may vary with the time. Thus, the total acid produced from the moment of inoculation at zero time to the time, t, is given by the integral,

$$A = \int_0^t \Phi \cdot C \cdot dt \qquad (1)$$

Since the population and acid production of a normal culture are seldom limited by the available food supply it is customary to assume that accumulation of metabolic products has an inhibiting effect upon cell growth and the ability of the cell to convert carbohydrates into acid. These two cellular functions, although subject to the relation given above, should be considered separately.

In our first paper (1935) a comparison was made between the acid production of *L. acidophilus* cultures grown without pH control and at various pH values. When the pH was controlled at the favorable value of 6.0 the acid production was 200 per cent greater than in normal growth. This observation suggests that the hydrogen ion is one of the metabolic products exerting

an inhibiting effect upon either C or Φ, or both, although from measurements of A alone we were unable to choose between these alternatives. Our recent measurements of acid production have, therefore, been accompanied by population studies.

Typical results from our more recent experiments are shown in figures 4 and 5. In these figures the times, in hours, from the moment of inoculation are plotted as abscissae while the ordinates

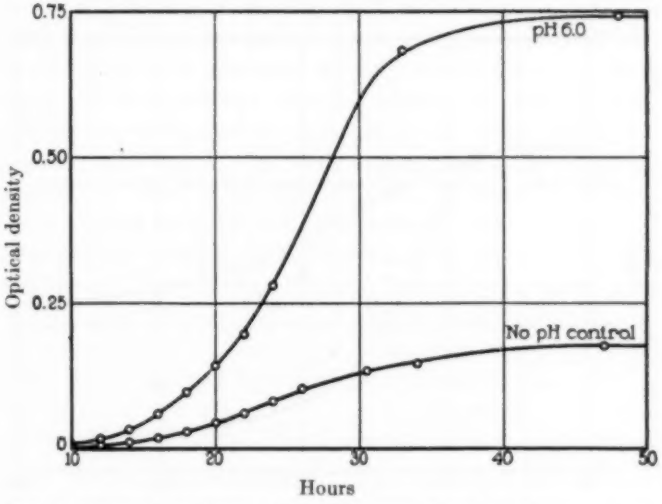


FIG. 5. CHANGE OF OPTICAL DENSITY, WITH TIME, OF CULTURES OF L. ACIDOPHILUS GROWN WITH AND WITHOUT pH CONTROL

are, respectively, the acid production in milli-equivalents per liter of medium and the optical density.[2]

It may be seen from figure 4 that, with pH control, the acid production at 50 hours has been increased 800 per cent over that of a culture in the same medium but without pH control. Moreover, this ratio was maintained until the experiments were

[2] It has been shown by Longsworth (1936) that for cultures of *L. acidophilus* grown with pH control measurements of optical density are proportional to the plate counts over an interval in which more than 90 per cent of the total growth occurs.

stopped at 100 hours, at which time the acid production was 77.5 milli-equivalents with normal growth and 712 with pH control. The latter value corresponds to 80 per cent conversion, computed as lactic acid, of the available sugar. The much larger increase resulting from pH control observed in these experiments than in the experiments described in our first paper can be attributed to the relatively rich medium employed in the more recent work.

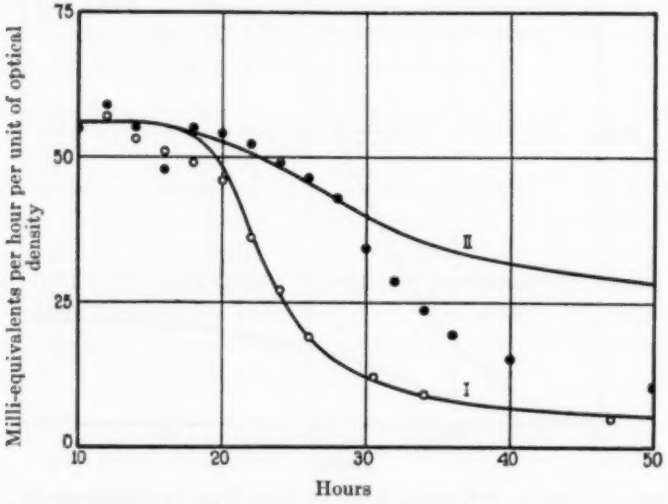


FIG. 6. RATE OF ACID PRODUCTION PER UNIT OF OPTICAL DENSITY OF A NORMAL CULTURE AND OF ONE GROWN AT pH 6.0

The culture grown with pH control also develops a much larger population, as measured by the optical density, than in normal growth. As shown in figure 5, the increase amounts to about 300 per cent at 50 hours. Plate counts were also made at intervals throughout these experiments and yield curves, not shown, similar to those of figure 5, except that the increase in the plate count due to pH control is 200 per cent. The difference between this increase and the four-fold change observed in the optical density, figure 5, is due to the fact that there is a larger proportion of short chains and individual cells in a normal culture whose

pH has attained low values, as has recently been shown by Longsworth (1936).

From the data represented by figures 4 and 5 values of ψ, the rate of acid production per *unit of optical density*,[3] may be computed. The results of these computations are plotted as ordinates against the time, in hours, as abscissae in figure 6, the open circles representing the values in normal growth and the solid circles corresponding to growth with pH control. In the early stages of growth, before the accumulation of appreciable concentrations of metabolic products, the values of ψ are approximately constant and independent of pH control. In the normal culture, however, ψ begins to decrease much earlier than in the culture at constant pH. It thus appears that the effect of pH control upon ψ is to delay the inhibiting action of the metabolic products on this property of the bacterial cell.

Although the hydrogen ion concentration (H^+) has been considered the most important limiting factor in the lactic fermentation (Rahn (1932)) evidence has accumulated to the effect that the concentration of lactate ion (L^-) is also a factor. These two variables are connected by the mass action relation

$$(H^+)(L^-) = K_{HL}(HL) \tag{2}$$

in which the ionization constant has the value of 1.55×10^{-4} (Borsook, Huffman, and Liu (1933)). The concentration of the undissociated acid (HL) is thus proportional to the product of both of these factors and has, as a matter of fact, been considered

[3] In our second paper, and in the formulation of equation 1 of the present paper, the unit of population used in the definition of ϕ was determined from plate counts, C. Since the optical density, D, may, as already mentioned, be taken as a measure of the population a quantity ψ, defined by the relation

$$\psi = \frac{dA/dt}{D}$$

will differ, in most instances, from ϕ by a constant factor. The quantities ϕ and ψ have essentially the same significance as the "fermenting capacity per cell" discussed by Rahn (1932). Equation 1 may thus be rewritten as

$$A = \int_0^t \psi \cdot D \cdot dt \tag{1'}$$

one of the chief inhibitors of growth and metabolism by Rogers and Whittier (1928) and others. In normal growth the production of lactic acid by the culture increases the values of both (H^+) and (L^-) whereas in growth at constant pH the former does not change. Undissociated lactic acid thus accumulates in both normal cultures and those grown with pH control, but more rapidly in the former case. The value of (HL) for either case may be computed as follows. The observed quantities, which are the titratable acid, A, and the pH, are related to (H^+) and (HL) by the equations,

$$(HL) + (L^-) = A \tag{3}$$

$$pH = \log \frac{1}{(H^+)} \tag{4}$$

Elimination of (L^-) between equations 2 and 3 yields, for the concentration of undissociated lactic acid,

$$(HL) = \frac{(H^+)}{K_{HL} + (H^+)} A \tag{5}$$

It was of interest to determine whether there is a simple relation between ψ and (HL) valid for the normal growth which also holds for growth with pH control. Among the many possible equations the following

$$\frac{\psi}{\psi_0} = \frac{1}{1 + a(HL)} \tag{6}$$

describes the change of ψ with the accumulation of lactic acid during normal growth. In this expression ψ_0 is the value of ψ for a young culture and a is an empirical constant with a value of 350. The agreement, which is within the experimental error, between the observed and computed values is shown in figure 6 in which curve I is a plot of equation 6. In obtaining this curve the values of (HL) were computed, using equations 4 and 5, from determinations of pH and A for the normal culture. The scattering of the points in the early stages of growth is due to the fact that the rate of acid production is low and the ψ values are therefore relatively inexact.

Using the same values of ψ_0 and a, but computing (HL) from the acid production data for the culture grown at pH 6.0, curve II of figure 6 was obtained. It may be seen that the observed values, indicated by the solid circles, follow curve II for a considerable part of the growth period. It must be recalled, however, that metabolism, as measured by the acid production, is much more rapid in the culture at pH 6.0 than in normal growth. Consequently by the time marked deviations from curve II are observed changes in the substrate, other than the accumulation of undissociated lactic acid, have probably occurred. These can affect the value of ψ.

Among such changes are increases of the salt concentration and depletion of the nutrient material. In normal growth the salt concentration retained its initially low value throughout the experiment while at constant pH the concentration of sodium lactate increased to a value of 0.7 N at 100 hours. Moreover, without pH control only 8 per cent of the sugar was utilized so that its concentration remained essentially constant. At pH 6.0, however, 80 per cent of this material was converted. Consequently these changes, which are relatively unimportant in normal growth, may affect the value of ψ in growth at constant pH and their influence was not considered in equation 6. Winslow (1934) has shown that salt concentrations of the magnitude developed in growth at constant pH have an inhibiting effect upon bacterial growth and metabolism. Moreover, although most enzyme reactions are independent of the concentration of substrate until that is reduced below a critical value, the disappearance of the sugar in the culture medium must ultimately reduce ψ to zero. It is not surprising, therefore, that the relatively simple relation represented by equation 6 can account for the changes in ψ that are observed in cultures grown at pH 6.0 for only the first portion of the growth period.

In conclusion, then, it would appear that accumulation of undissociated lactic acid (HL), or in other words an increase of the ionic product $(H^+)(L^-)$, has an inhibiting effect upon both the growth and metabolism of *L. acidophilus*. If one of the factors (H^+) is held at an appropriate value this effect is very

much reduced. However, even with pH control, alterations of the substrate and accumulation of other metabolic products ultimately have inhibiting effects.

SUMMARY

A method for the production of oxygen-free mixtures of carbon dioxide and nitrogen has been developed. With the aid of these mixtures and an apparatus for automatic pH control we have studied, at pH 6.0, the growth, acid production, and apparent oxidation-reduction potentials of cultures of *Lactobacillus acidophilus* under anaerobic conditions. The observation that carbon dioxide is essential for the growth of this organism has been confirmed.

Apparent oxidation-reduction potentials, E_h, in these cultures have been obtained with the aid of gold and platinum foil electrodes. Of these the gold electrode appears to respond more readily to changes in the oxidation-reduction balance of the system. During anaerobic growth the E_h (gold) drops rapidly to a minimum of about -190 mv., then increases to a value of about -150 mv. after which it slowly returns to the minimum value, which appears to be a characteristic of the bacteria, having been obtained in all anaerobic cultures. The relation of these anaerobic E_h measurements to possible metabolic processes in the culture has been discussed.

Using a more suitable medium than in previous experiments the measurements of growth and acid production at a constant pH of 6.0 have been compared with similar measurements in normal cultures. Control of the pH resulted in a four-fold increase of the bacterial population and a nine-fold increase of the acid production.

REFERENCES

BARRON, E. S. G., AND HASTINGS, A. B. 1934 Jour. Biol. Chem., **107**, 567.

BORSOOK, H., HUFFMAN, H. M., AND LIU, Y. P. 1933 Jour. Biol. Chem., **102**, 449.

CAVEN, R. M., AND SAND, H. J. S. 1911 Jour. Chem. Soc., **99**, 1359.

CLIFTON, C. E., CLEARY, J. P., AND BEARD, P. J. 1934 Jour. Bact., **28**, 541.

COULTER, C. B. 1928–29 Jour. Gen. Physiol., **12**, 139.

GILLESPIE, R. W. H., AND RETTGER, L. F. 1936 Jour. Bact., **31**, 14.

GLADSTONE, G. P., FILDES, P., AND RICHARDSON, G. M. 1935 Brit. Jour. Exp. Path., **16**, 335.
HEGARTY, C. P. 1936 Jour. Bact., **31**, 18.
HEWITT, L. F. 1935 Oxidation-Reduction Potentials in Bacteriology and Biochemistry. 3rd edition. London County Council.
LONGSWORTH, L. G. 1936 Jour. Bact., **32**, 307.
LONGSWORTH, L. G., AND MACINNES, D. A. 1935 Jour. Bact., **29**, 595.
LONGSWORTH, L. G., AND MACINNES, D. A. 1936 Jour. Bact., **31**, 287.
MICHAELIS, L., AND FLEXNER, L. B. 1928 Jour. Biol. Chem., **79**, 689.
RAHN, O. 1932 Physiology of Bacteria, p. 144. Philadelphia, Blakiston.
ROGERS, L. A., AND WHITTIER, E. O. 1928 Jour. Bact., **16**, 211.
WINSLOW, C. E. A. 1934 Quart. Rev. Biol., **9**, 259.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

SEVENTH MEETING, NEW YORK CITY BRANCH

TEACHERS' COLLEGE, COLUMBIA UNIVERSITY, OCTOBER 13, 1936

SERGEI NIKOLAEVITCH WINOGRADSKY, A GREAT MICROBE HUNTER. *Selman A. Waksman*, New Jersey Agricultural Experiment Station, New Brunswick.

S. N. Winogradsky was born on September 1, 1856, in Kiev, Russia, of a rich and aristocratic family. Educated in the Universities of Kiev and St. Petersburg, he soon became attracted by the study of the morphology and physiology of bacteria. This was followed by his epoch-making work on the autotrophic bacteria, including sulfur-oxidizing and iron bacteria, and finally nitrifying bacteria. These investigations were carried out in the laboratory of De Bary in Strasbourg and at the University of Zurich (1885–1891). In 1891, Winogradsky returned to St. Petersburg to organize a division of general microbiology at the newly established Institute of Experimental Medicine. He was later made director of the institute and editor of Archives of Biological Sciences. The study of the non-symbiotic nitrogen-fixing and pectin-decomposing bacteria completed the first period of his scientific career. In 1905 he retired to his estate in Podol to engage in practical agriculture. The Russian revolution forced him to leave his native country and seek refuge first in Yugoslavia, then at the Pasteur Institute in Paris, where he established a division of soil microbiology. In his new laboratory at Brie-Comte-Robert, he devoted himself to the study of the microbiological population of the soil, including methods of studying soil microorganisms, the nature of cellulose-decomposing, nitrogen-fixing and nitrifying bacteria. At the age of 80, Winogradsky is still as active and vigorous as ever before. He is now busily engaged in completing some of the investigations begun in his youth.

WINOGRADSKY'S DISCOVERY AND STUDIES OF AUTOTROPHIC BACTERIA. *R. L. Starkey*, Department of Soil Microbiology, New Jersey Agricultural Experiment Station, New Brunswick.

Winogradsky evidenced great interest in several groups of bacteria having specific physiological characteristics, but his most significant contribution is the discovery of the group of autotrophic bacteria which he accurately portrayed and some representatives of which he was the first to observe. He discovered the mechanism by which the sulfur and iron bacteria obtain energy for growth and received acclaim from the scientific world for his brilliant studies with the nitrifying bacteria. The autotrophic organisms were characterized by him as follows: (1) Their development in nature occurs only in strictly elective mineral media

which must contain the specific oxidizable inorganic substances. (2) Their existence is dependent upon the presence of these substances which undergo oxidation through their life processes. (3) This oxidation process is their sole source of energy. (4) They require no organic nutrients either as plastic material or as a source of energy. (5) They have little or no ability to utilize organic substances, even becoming injured by them during their growth. (6) They use carbonic acid exclusively as a carbon source, which is assimilated through chemosynthesis. Present knowledge of the specific physiology of the autotrophic bacteria conforms closely to the pattern proposed by Winogradsky nearly fifty years ago.

STUDY OF THE TOXINS OF THE PNEUMOCOCCUS. *A. F. Coca, P. A. Little, B. M. Lyon and E. F. Voigt*, Lederle Laboratories, New York.

I. Filtrates of the pneumococcus show toxicity for rabbit (skin reaction and temperature rise); mouse (lethal); horse (temperature rise). Repeated injection of the filtrate in animals results in the relatively rapid production of antitoxin.

II. Three methods of standardization of the antitoxin have been tried. The first of these was by mouse protection (antitoxic). Antitoxic protection could be demonstrated but the results were quantitatively irregular. The antihemolytic test and the titration according to the point of optimal flocculation gave quite constant and concordant results.

Both of two type III strains of pneumococcus recently obtained from Dr. Goodner produced the most toxic filtrates yet studied. One of these strains is pathogenic for rabbits; the other is not. Both are pathogenic for mice.

Confirming earlier results in human beings (skin-tests), the toxic substance in the type III filtrate has been quantitatively separated from the capsular polysaccharide.

THE STANDARDIZATION OF THE TOXINS AND ANTITOXINS OF THE PNEUMOCOCCUS. *Drs. A. F. Coca and P. A. Little*, Lederle Laboratories, New York.

ATTEMPTS TO INCREASE THE HEAT RESISTANCE OF BACTERIAL SPORES[1]

FRED T. WILLIAMS

Department of Agricultural Bacteriology, University of Wisconsin

Received for publication July 6, 1936

Bacteria are conspicuous among spore-forming plants for the great resistance of their spores to drying, to dyes, to germicidal chemicals and particularly to heat. Heat resistance is at times almost unbelievable and it is this property of bacterial spores which makes their destruction by heat sterilization, as in the processing of canned food, so great a problem. A great deal of work on heat resistance has therefore been done, and it has been found that the resistance of any particular species is not fixed but varies with several factors. The conditions under which the spores are produced as well as their age and previous treatment apparently are important. The concentration of spores, pH of the substrate in which they are heated, and the presence or absence of protective colloids or salts are also factors. These points are discussed in the reports of Burke (1919), Bigelow and Esty (1920), Weiss (1921), Esty and Meyer (1922), Magoon (1926a), Curran (1935), and others. Esty and Meyer have also noted that spores of different strains of the same organism vary widely in their heat resistance and that all of the spores in a particular suspension do not have the same resistance, a small percentage apparently being capable of surviving heating for much longer periods than the majority. A recent confirmation of this fact of individual variation is found in the report of Yesair and Cameron (1936) in which they showed difference in specific gravity between the more resistant and the less resistant spores of a population. This variability has suggested to several

[1] Part III of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The University of Wisconsin, 1936.

workers that it may be possible to increase the heat resistance of the spores of bacteria by a process of artificial selection. Thus, by heat-treating a spore suspension so that only the more resistant spores survive and are allowed to produce the next culture generation, it was thought that a strain of the organism could be developed with greatly enhanced spore resistance. Bigelow and Esty (1920), Magoon (1926b), O. B. Williams (1929), and Walsh (1932) have reported marked increases in spore resistance of several organisms as obtained by selection. Magoon claimed the most remarkable increase, having changed the heat resistance of spores of *Bacillus mycoides* from 2 to over 50 minutes at 100°C. Sommer (1930) was unable to increase the thermal death time of spores of *Clostridium botulinum* by a similar method.

The implications of such a modification of heat resistance are of great practical and theoretical interest. It, therefore, appeared desirable to attempt to duplicate Magoon's work using several different organisms.

Furthermore, it is not clear in the literature whether the authors consider the increased resistance of the culture derived by heating to be a gradual, almost evolutionary *change* of the spore resistance, or merely a *shift* in the population by which the few exceptionally resistant individuals become predominant. To simplify the experiment and to test the theory of adaptation to heat as the cause of increased resistance, single cell cultures were used in this study.

CULTURES EMPLOYED

Single cell isolations were made of the following organisms: *Bacillus mycoides*, *Bacillus graveolens*, *Bacillus subtilis*, *Clostridium Pasteurianum*, *Clostridium butylicum*, *Clostridium acetobutylicum*, and *Clostridium sporogenes*. The aerobic species (*Bacillus*) were originally secured from stock cultures carried in our laboratory. They have been carried on nutrient agar medium for a number of years with frequent transfers to fresh medium. The anaerobic species (*Clostridium*) are maintained in our laboratory as dry spore stock on sterile soil and the single

cell isolations were made from cultures which were started from these soil stocks.

After the single cell isolations were made, soil stock cultures were prepared of each culture and set aside until the start of the experiment. These stock cultures were saved to provide a check to be run at the end of the experiment, in case an apparent change in the thermal death time of the organism should occur during the process of selection.

MEDIA AND INCUBATION

Spore crops of the aerobic organisms were obtained by cultivation on nutrient-agar bottle-slants at 28°C. These were subcultivated on nutrient broth at 37°C. after the heat treatment. Spore crops of *Cl. sporogenes* were obtained from growth on liver extract agar; spores of the other anaerobic species from the surface of an agar medium containing 1 per cent Difco tryptone and 0.5 per cent glucose. All anaerobic species were subcultivated in 5-per-cent corn-mash tubes containing 1-per-cent powdered liver (McClung and McCoy, 1934). All incubations of the anaerobes were carried out at 37°C. in a sealed jar, made anaerobic by the respiration and fermentation of moist oats.

The incubation period varied from 5 to 30 days, both for raising spore crops and for noting viability after heat treatment. Inasmuch as the experiments were carried out over a period of nine months it was not always convenient for incubation periods to be of uniform duration. This was not considered necessary, however, because the final tests of the culture derived by selection were compared with the original cultures which were run in parallel at the conclusion of the experiment.

PREPARATION OF SPORE SUSPENSIONS AND HEAT TREATMENT

The spores were washed from the agar slants with sterile water and shaken with glass beads to break up clumps. The numbers of spores in these suspensions were counted by means of the Petroff-Hauser counter and then diluted in a sterile M/15

phosphate buffer solution (pH 7.0) until the final concentration was approximately 10,000,000 spores per cubic centimeter. One-cubic-centimeter amounts of suspension were then sealed into small soft glass ampules and the heat treatment made after the method of Esty and Williams (1924). An electrically heated oil bath with a motor driven turbine and a thermo-regulator was employed. The temperature was not allowed to vary more than 0.5°C. After the heat treatment the suspensions were immediately cooled and subcultivated to tubes of nutrient broth or corn liver medium. The culture developing from the spores showing the highest resistance was inoculated to an agar bottle slant for the production of a fresh crop of spores. This procedure was carried out for eight or ten successive times with each culture studied. Occasionally in the tests, contamination was noted. In such cases the contaminated culture was promptly discarded.

EXPERIMENTAL RESULTS

A preliminary determination of the thermal death times of the several organisms was made in order to determine the temperatures which offered the most convenient death times. Thus, throughout the work to be described, the following temperatures were used for heating the spore suspensions of the several organisms: *B. subtilis*, 115°C.; *B. graveolens*, 100°C.; *B. mycoides*, 100°C.; *Cl. Pasteurianum*, 100°C.; *Cl. acetobutylicum*, 100°C.; *Cl. butylicum*, 90°C.; and *Cl. sporogenes*, 90°C.

No difficulty was experienced in securing uniform spore suspensions except in the case of *B. mycoides*. A considerable number of vegetative cells were always present in the suspensions of anaerobes, however. All of the results are presented in figure 1. The series number, the age of the spore culture from which each of the suspensions was prepared, and the time that each suspension resisted the specified temperatures under the conditions described above are indicated. All tests were run in duplicate except the final series and the parallel check series for the original single cell culture; these were run in quadruplicate. Solid bars in the figures indicate uniform growth in all tubes out to the time limit of survival. Breaks or white spaces in the bars

indicate the times at which the familiar skip-stop phenomenon occurred and no growth in such tubes was noted. The survival

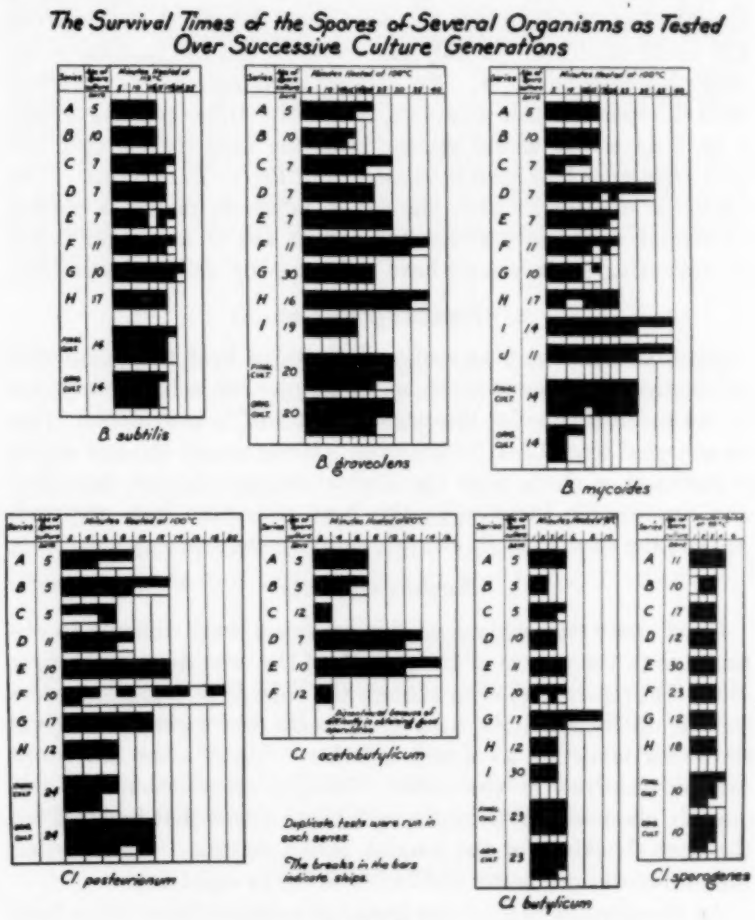

The Survival Times of the Spores of Several Organisms as Tested Over Successive Culture Generations
B. subtilis
B. graveolens
B. mycoides
Cl. pasteurianum
Cl. acetobutylicum
Cl. butylicum
Cl. sporogenes
Duplicate tests were run in each series.
The breaks in the bars indicate skips.

Fig. 1

times indicated are maximum since in every series the spores were heated for a longer period than that which would allow them to survive and germinate.

Bacillus subtilis

Inspection of figure 1 shows that the survival time of this organism at 115°C. under the conditions described, varied from 12½ to 20 minutes. In only one instance did the familiar skip-stop phenomenon occur. No delayed germination was observed with the spores of this organism, all positive tubes growing within 3 or 4 days. It would appear from the final results that the heat resistance had been increased from 12½ to 15 minutes. The variation observed within the series, however, makes it appear somewhat doubtful whether the results are of any significance as indicating an increased heat resistance by artificial selection.

Bacillus graveolens

Even though conditions of uniformity of heat treatment and cultivation were maintained as far as possible a large variation in the survival time for this organism at 100°C. is evident. This time varied from 15 to 35 minutes. However, at the end of the experiment, a check with the original culture did not show that any appreciable increase in the heat resistance had occurred. As was the case with *B. subtilis*, no dormancy was noted.

Bacillus mycoides

Good spore suspensions of this organism were difficult to obtain due to the nature of the growth of the organism on the agar slant. Furthermore, even though the clumps of organisms were broken up by agitation with glass beads the spores themselves showed a tendency to clump together. Many skips and stops can be observed in the data. Delayed germination was frequently observed, particularly with those spores that had resisted the heat shocking for the longest period of time. Germination sometimes did not occur until after seven or eight days.

An apparent increase in the heat resistance of these spores from 10 to 25 minutes is indicated. The same criticism may be applied here as in the case of *B. subtilis*; namely, that some other factor than simple selection may be concerned, because of the large variation observed between the several series. Apparent decreased resistance is noted in successive series as well as increased resistance.

Clostridium Pasteurianum

A large number of skips and stops occurred with this organism in the different series. This may be explained in large part as due to the difficulty often encountered in the cultivation of this anaerobe. The corn-liver mash that was used offers a good medium for growth but germination in it does not always occur even upon the addition of sterile reduced iron.

At the final test, it was found that the original organism had a greater resistance than that which had been passed through the series. The selection in this case appeared to work negatively.

Clostridium acetobutylicum

It was difficult to secure good spore crops with this organism. Often no spores were produced at all on the agar medium. In view of this fact, work was not completed in this instance. The graph shows, however, the variation in thermal death times exhibited by this organism. As in the case of *Cl. Pasteurianum*, it is difficult to cultivate this organism and the large variation observed may be at least partially explained on this basis. Delayed germination was observed up to a period of 10 days.

Clostridium butylicum

The survival time for this organism evidently lies in the range of 3 or 4 minutes at 90°C. In one case it appeared to be 8 minutes. The final test shows, however, that no significant change had occurred in the resistance of the organism during the selection. Delayed germination was not observed.

Clostridum sporogenes

Examination of the graph shows that the thermal death times of this species were very constant throughout the experiment. No alteration in the heat resistance of the spores was detected during the run nor in the final check against the original. No delayed germination by these spores was evidenced.

DISCUSSION

Inspection of the graphs shows a considerable variation in the heat resistance of the spores of a single species of organism. Except in the case of *Clostridium sporogenes*, large variations in the thermal death times of the several organisms are apparent as determined from time to time under carefully controlled conditions. Contrary to the results of Magoon, these variations did not always occur in the same direction and it was found necessary to compare at the end of the tests the organism that had passed through heat series directly with the original organism. A significant increase in the heat resistance by this process of artificial selection was obtained only in the case of *Bacillus mycoides*. This is the organism on which Magoon reported the increase from 2 to over 50 minutes at 100°C. in 6 selective transfers. In the experiment here reported, an increase from 10 to only 25 minutes was observed after 10 selective heat series. The tendency of spore suspensions of this organism to clump may be a factor in the apparent increase in resistance.

Delayed germination was noted with several organisms; however, no attempt was made to determine a maximum time for the germination of these cultures. The occurrence and extent of this phenomenon has been adequately reported for several organisms by numerous workers (Burke, 1923; Dickson et al., 1925; McCoy and Hastings, 1928).

Spores of varying ages were used in the different series and no direct correlation could be observed between the age of the spores and their heat resistance. This has been reported to be a factor by Weiss, Esty and Meyer, Magoon and Curran. Minor variations apparently occur even though conditions are duplicated as closely as possible.

SUMMARY AND CONCLUSIONS

1. An attempt was made to increase by artificial selection the heat resistance of the spores of the following organisms: *Bacillus subtilis, Bacillus graveolens, Bacillus mycoides, Clostridium Pasteurianum, Clostridium acetobutylicum, Clostridium butylicum,*

and *Clostridium sporogenes*. Single cell cultures were used so that any increase obtained could be interpreted as an actual change in resistance produced by adaptation.

2. After 8 or 10 selective series with each organism, no significant increase was observed except in the case of *Bacillus mycoides*. The apparent increase in this instance was from 10 to 25 minutes at 100°C.

3. Even though all known factors were carefully controlled in the production and heat treatment of the spores, wide variations occurred in the heat resistance as tested at different times.

4. The age of the spore cultures varied from 5 to 30 days. No correlation could be observed in any case between the age of the spores and their heat resistance. The final tests upon original and derived cultures were upon spores of comparable age so as to eliminate the age variable.

5. Delayed germination of spores was noted in several instances.

REFERENCES

Bigelow, W. D., and Esty, J. R. 1920 Jour. Infect. Diseases, **27**, 602.

Burke, Georgina S. 1919 Jour. Amer. Med. Assoc., **72**, 88.

Burke, Georgina S. 1923 Jour. Infect. Diseases, **33**, 274.

Curran, Harold R. 1935 Jour. Infect. Diseases, **56**, 196.

Dickson, Ernest C., Burke, Georgina S., Beck, Dorothy, and Johnston, Jean 1925 Jour. Infect. Diseases, **36**, 472.

Esty, J. R., and Meyer, K. F. 1922 Jour. Infect. Diseases, **31**, 650.

Esty, J. R., and Williams, C. C. 1924 Jour. Infect. Diseases, **34**, 516.

Magoon, C. A. 1926a Jour. Bact., **11**, 253.

Magoon, C. A. 1926b Jour. Infect. Diseases, **38**, 429.

McClung, L. S., and McCoy, Elizabeth. 1934 Jour. Bact., **28**, 267.

McCoy, Elizabeth, and Hastings, E. G. 1928 Soc. Exper. Biol. and Med. Proc., **25**, 753.

Sommer, E. W. 1930 Jour. Infect. Diseases, **46**, 85.

Walsh, T. Crosbie 1932 Food Manufacture, **7**, 107.

Weiss, Harry 1921 Jour. Infect. Diseases, **28**, 70.

Williams, O. B. 1929 Jour. Infect. Diseases, **44**, 421.

Yesair, J., and Cameron, E. J. 1936 Jour. Bact., **31**, 2.

GLYCEROL AND CARBOHYDRATE UTILIZATION BY MYCOBACTERIUM TUBERCULOSIS

A. G. WEDUM

Department of Bacteriology, Northwestern University Medical School, Chicago

Received for publication, July 7, 1936

The experiments reported here had two objectives. The first was a study of the fate of glycerol in fluid medium cultures of *Mycobacterium tuberculosis*, the rapidity of its utilization and its end products. This has been discussed by Wells and Long (1932) and Loebel, Shorr, and Richardson (1933). The second objective was to confirm and extend reports regarding the utilization of various carbohydrates.

The glycerol metabolism of the tubercle bacillus is difficult to examine directly because of the lack of simple and accurate methods of glycerol analysis. Siebert (1909) and Tiffeneau and Marie (1912) found that most of the glycerol disappeared from broth media during growth. Frouin and Guillaumie (1926) obtained similar results in a synthetic medium. Dingle and Weinzirl (1932) used a synthetic medium and favor the view that glycerol is completelv oxidized to carbon dioxide and water. Model, Gurjeff, and Pirogoff (1933) found that about one-tenth of the glycerol remained in the culture medium after 30 days.

In the present work the synthetic medium of Long and Seibert (1926) or a variation thereof was employed. The only organic matter present besides glycerol was 0.5 per cent asparagin and 0.5 per cent ammonium citrate. It was therefore possible to follow the course of glycerol utilization by a simple oxidation procedure in which all the organic matter present analyzed as glycerol. Model *et al.* (1933) reported the use of this method shortly after the present study was begun but only a few determinations are given.

The carbohydrate metabolism of *M. tuberculosis* is unusual in

that little or no acid is produced. The end products according to Merrill (1930, 1931) are chiefly carbon dioxide and water. Consequently only analytical methods are satisfactory for studies of its carbohydrate metabolism. Reports of the non-utilization of various carbohydrates as determined by acid-indicators or other indirect methods have been published by Weinzirl and Knapton (1927), Henley (1929), Merrill (1931), Model (1933), Loebel, Shorr, and Richardson (1933). Other references may be found in the literature cited. The findings of those investigators who have employed the more exact method of analysis for reducing sugars are given in table 6.

METHODS

Organisms. The human tubercle bacilli used were (1) the well-known virulent "H 37," (2) a virulent culture known as "G I," isolated in 1931 by Dr. F. D. Gunn from a fatal human case, and (3) "597," an avirulent strain obtained from the American Museum of Natural History sometime before 1914. The bovine strains were (1) a strain from the National Tuberculosis Association, (2) "BAI" obtained several years ago from the Bureau of Animal Industry, and (3) a BCG culture sent by Dr. W. H. Park in 1931. The avian strain used was from the National Tuberculosis Association.

Glycerol methods. Usually, about 50 or 100 cc. of autoclaved Long's medium were poured respectively into sterile 125 or 250 cc. Erlenmeyer flasks. A bit of sterile cotton or cork served as a float for the initial inoculum. Variations from this procedure are indicated in the tables. The flasks were incubated 4 to 7 days to insure sterility.

Evaporation was minimized by paraffining the cotton stoppers or by using in each flask (in addition to the usual cotton plug cut short and singed) a sterile one-hole rubber stopper containing a short piece of 1 mm. bore glass tubing. Incubation was at 37°C.

The hydrogen-ion concentration was determined colorimetrically, using brom thymol blue and brom cresol purple as indicators. In the earlier experiments the analyses were made on samples obtained by pipette directly from the flask. In later

experiments the material for analysis was first passed through filter paper. There was no significant difference in the results. Ammonia nitrogen was determined on 2 cc. samples by the usual aeration method and total nitrogen by the method of Koch and McMeekin (1924). Whenever the flasks were opened, a loopful or several drops of the culture were transferred to 1 or 2 glucose broth tubes as a test for the presence of contaminating bacteria.

Glycerol was determined by oxidation with potassium dichromate as used in the Methods of Analysis of the Association of Official Agricultural Chemists (1925) for the analysis of vinegar. The procedure was modified and adapted to the present study as follows. Filter the cultures through fine filter paper. In a 100 cc. volumetric flask place a 2 cc. sample. Add 25 cc. of 1.52 N potassium dichromate. Add, with cooling, 20 cc. concentrated sulphuric acid. Place in a boiling water bath 20 minutes. Cool 10 minutes. Make up to 100 cc. with distilled water and mix well. Take a 5 cc. sample and titrate with N/10 sodium thiosulphate, in the presence of 5 cc. concentrated hydrochloric acid, using a spatula point of potassium iodide, with starch as an indicator. The percentage of "glycerol" in the original sample may be obtained from the formula $(19 - x)\ 0.658$ in which 19 is the number of cubic centimeters of N/10 dichromate in the blank titration flask, x is the number of cubic centimeters of N/10 thiosulphate used in the titration, and 0.658 is the percentage of glycerol represented by each cubic centimeter of N/10 dichromate utilized.

Since all organic matter present analyzes as glycerol, one might think that tuberculo-protein in solution, asparagin, ammonium citrate, and various decomposition products would seriously interfere with the accuracy of the method. Actually they do not. The 0.5 per cent asparagin present in the uninoculated medium analyzes as about 0.19 per cent "glycerol" and the 0.5 per cent ammonium citrate as about 0.25 per cent. When compared to the amount of initial glycerol, the dissolved protein in the medium at the end of the incubation period is negligible (table 5).

Carbohydrate methods. The basic media were sterile beef heart infusion broth containing 0.5 per cent glycerol, and Long's

medium containing 2.5 per cent glycerol. To these were added sufficient sterile Berkefeld-filtered 10 per cent carbohydrate solutions to make concentrations of about 0.45 per cent for monosaccharides and 0.70 per cent for disaccharides. The media were dispensed in tubes 15 to 20 x 150 mm., 8 to 10 cc. per tube, and incubated 3 days to insure sterility. Small pieces of sterile cork served as floats for the inocula. Tubes were inoculated in duplicate. Incubation was at 37°C. for 4 months. Evaporation was minimized by the use of rubber stoppers as in the glycerol experiments. The initial pH of the broth media was 6.7 to 7.1 and of the Long's media 7.2 to 7.5. Analyses were made for reducing sugars as described by Wedum (1936). The pH was determined colorimetrically.

RESULTS

Glycerol. Table 1 shows what may be expected of the accuracy of the "glycerol" analyses. By careful work one can generally stay within a variation of 0.10 per cent glycerol. Asparagin had about one-third the reducing power of glycerol and ammonium citrate one-half. Inorganic ammonium salts had no effect. When sterile Long's medium was treated with an equal volume of 20 per cent trichloroacetic acid and filtered, the accuracy of the analysis was unaffected. From table 1 it can be seen that, when the actual glycerol in the medium is the usual 6.25 per cent (by weight), the 0.52 per cent which represents the other organic constituents is of comparatively little significance in the analysis. The H 37 cultures in table 1 were grown in a Long's medium of 2.78 per cent glycerol. This was done in the hope that the residual organic matter in the medium would be less at the end of the experiment than if the usual 6.25 per cent glycerol were used. Such was not the case (table 3).

Table 2 indicates the degree of utilization of "glycerol" by selected strains under various conditions. Human, bovine, BCG, and avian strains utilized practically all the oxidizable organic matter present in the medium. It is interesting that the residual oxidizable material in the culture medium is so uniform in amount regardless of the strain of organism (except the aviru-

lent human 597), and regardless of the volume of fluid and initial concentration of glycerol, asparagin, and ammonium citrate.

TABLE 1

Accuracy of the method of glycerol analysis

SUBSTANCE ANALYZED	"GLYCEROL"	
	2 cc. Sample No. 1	2 cc. Sample No. 2
	grams per 100 cc.	*grams per 100 cc.*
H 37—192 day culture, Flask 1	0.46 0.43	0.46 0.43
H 37—208 day culture, Flask 2	0.44 0.32	0.40 0.30
H 37—192 day culture, Flask 3	0.41 0.40	0.30 0.26
Control Flask 1	2.51 2.51	
Control Flask 2	2.70 2.70	
Blank 1, put through the same procedure	0.10 0.13	
Blank 2, put through the same procedure	0.13 0.07	
100 mgm. egg albumin,* sample 1	4.04 4.00	
100 mgm. egg albumin,* sample 2	4.04	
Ammonium citrate, 5 per cent in water	2.60	
Asparagin, 2 per cent in water	0.72 0.76	
Diammonium phosphate, 4 per cent in water	0.06	
Long's medium	6.65	
Long's medium; no glycerol, 0.5 per cent asparagin and citrate	0.52†	
Long's medium; no glycerol, no asparagin, 0.5 per cent citrate	0.30	

* Crystalline powdered ether dried.

† The synthetic medium of Model *et al.* (1933) is reported to analyze as only 0.132 gram oxidizable material per 100 cc. (in the absence of glycerol).

It should be kept in mind that the method of analysis was usually accurate within 0.10 per cent glycerol.

Table 3 corroborates the finding of Dingle and Weinzirl (1932)

that pathogenic human strains produce a terminal acidity and avirulent human strains a terminal alkalinity. But, if the glycerol content of the medium is reduced and the incubation is prolonged beyond the 70-day period of Dingle and Weinzirl, the culture of the pathogenic strain may become alkaline. Smith

TABLE 2

"Glycerol" utilization by human, bovine, and avian tubercle bacilli

STRAIN	INOCULATION DATE	AGE OF CULTURE	MEDIUM	INITIAL "GLYCEROL"	TERMINAL "GLYCEROL"	INITIAL pH	TERMINAL pH
		days		*grams per 100 cc.*	*grams per 100 cc.*		
G I	5/17/32	131	2	6.56	0.20	7.1	6.2
H 37	3/23/33	47	1	6.61	0.36	7.0	5.7
H 37	11/22/35	208	1	2.60*	0.34	7.5	7.3
H 37	11/ 6/35	192	1	2.78*	0.37	7.2	7.2
597	5/17/32	131	2	6.81†	0.00	7.1	8.4+
N. Tb. A. bovine	6/20/32	101	2	5.39‡	0.37	7.0	6.8
	2/ 3/33	63	1	7.45	0.34	6.9	6.7
	3/23/33	72	1	6.61	0.40	7.0	6.3
	5/17/32	94	2	6.56	0.24	7.1	5.9
	9/24/34	348	3	3.61§	0.34	7.2	7.2
BAI	5/17/32	131	2	6.81†	0.45	7.1	6.2
BCG	9/24/34	348	3	3.61§	0.37	7.2	7.2
Avian	5/17/32	131	2	6.56	0.42	7.1	6.0
Avian	5/17/32	131	2	6.81†	0.21	7.1	6.3

1 = 100 cc. Long's medium in a 250 cc. Erlenmeyer flask.
2 = 50 cc. Long's medium in a 125 cc. Erlenmeyer flask.
3 = 15 cc. Long's medium in a U-tube.

*0.2 per cent asparagin and 0.2 per cent ammonium citrate instead of the usual 0.5 per cent.

† Including 0.46 per cent glucose; terminal glucose (as reducing sugar) 0.03 per cent (597); 0.16 per cent (BA 1); 0.03 per cent (avian).

‡ Including 0.66 per cent glucose; terminal glucose 0.06 per cent.

§ 0.3 per cent asparagin and 0.3 per cent ammonium citrate instead of the usual 0.5 per cent.

(1910) found a similar tendency toward alkalinity in 2 per cent glycerol broth and believed that the glycerol was much more nearly exhausted from the medium than in 5 per cent glycerol broth. Such a view is probably incorrect inasmuch as table 3 shows that in either case in synthetic medium the amount of residual oxidizable matter is practically the same.

Figure 1 is a composite of 5 flasks repeatedly analyzed. It shows how closely the drop in pH corresponds during the period of maximum growth to the utilization of oxidizable material, chiefly glycerol. Graphs similar in appearance occur when the pH is correlated with the change in the total nitrogen and the ammonia content of the medium as has been done by Dingle and

TABLE 3

Terminal pH of virulent and avirulent human strains in Long's medium

STRAIN	NUMBER OF CULTURES	INOCULATION DATE	MEDIUM	AGE OF CULTURE	INITIAL "GLYCEROL"	AVERAGE TERMINAL "GLYCEROL"	INITIAL pH	TERMINAL pH
				days	*grams per 100 cc.*	*grams per 100 cc.*		
H 37	2	9/24/34	3	348	3.61*	0.62	7.2	7.2
H 37	6	11/22/35	1	208	2.60†	0.45	7.5	7.3
H 37	2	11/ 6/35	1	192	2.78†	0.44	7.2	7.1
H 37	3	3/23/33	1	47	6.61	0.57	7.0	5.7
G I	1	6/20/32	2	101	5.39‡	1.19	7.0	5.6
G I	1	5/17/32	2	131	6.56	0.20	7.1	6.2
G I	1	5/17/32	2	131	6.81§	0.65	7.1	5.9
597 (avirulent)	1	5/17/32	2	131	6.81§	0.00	7.1	8.4+

1 = 100 cc. Long's medium in a 250 cc. Erlenmeyer flask.

2 = 50 cc. Long's medium in a 125 cc. Erlenmeyer flask.

3 = 15 cc. Long's medium in a U-tube.

*0.3 per cent asparagin and 0.3 per cent ammonium citrate instead of the usual 0.5 per cent.

†0.2 per cent asparagin and 0.2 per cent ammonium citrate instead of the usual 0.5 per cent.

‡ Including 0.66 per cent glucose; terminal glucose 0.26 per cent.

§ Including 0.46 per cent glucose; terminal glucose 0.10 per cent (G 1), 0.03 per cent (597).

Weinzirl (1932) and discussed by Wells and Long (1932). The experiment was repeated with similar results.

The dichromate method of analysis would cause any organic acids present to analyze as glycerol. The fact that a decrease in the concentration of oxidizable material accompanies so closely the decrease in pH indicates that there is no appreciable accumulation of oxidizable organic acids. Although a small amount may accumulate during the period of maximum growth and be

utilized later, the results do favor the theory that the glycerol is either converted directly to carbon dioxide and water, or that the organic acids are consumed about as rapidly as they are produced. Support is thus gained for the view that the acidity is caused by a withdrawal of the alkaline constituents of the medium rather than by an increase in organic acid.

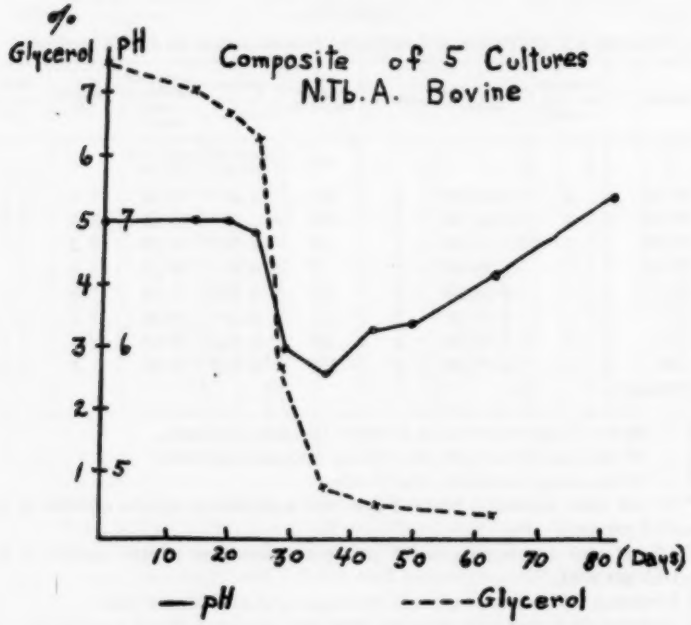


FIG. 1

The human strain H 37 presents a glycerol curve similar to that of the bovine type. Figure 2 represents one flask. Two other cultures of H 37 were similar in action. According to Model *et al.* (1933), autolytic processes probably account for the decreased acidity in the old cultures.

Table 4 gives in greater detail the results of analysis of the filtered culture medium after growth of H 37 for 192 days. In addition to the usual other constituents of Long's medium this

medium contained initially 2.6 per cent glycerol, 0.2 per cent asparagin, and 0.2 per cent ammonium citrate.

Table 5 indicates that very little of the residual organic matter analyzing as glycerol was protein in nature. The cultures of H 37 after 348 days in Long's medium containing 0.3 per cent asparagin and 0.3 per cent ammonium citrate were analyzed before and after treatment with (1) an equal volume of 20 per cent trichloroacetic acid, and (2) an equal volume of 20 per cent trichloro

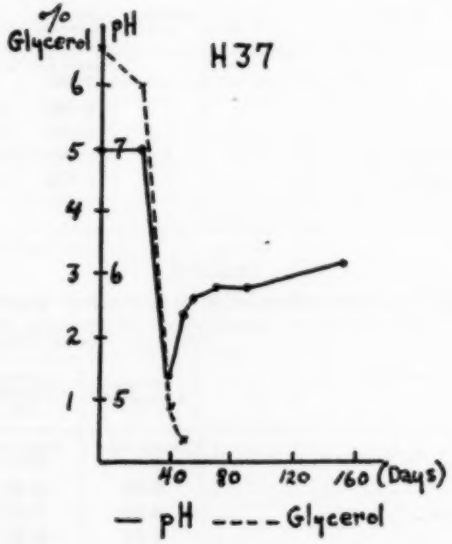


FIG. 2

acetic acid followed by an equal volume of a 10 per cent suspension of kaolin. These protein precipitants did not reduce the concentration of oxidizable material.

It is interesting to compare the data of tables 4 and 5 with the results of Dingle and Weinzirl (1932) and Model *et al.* (1933). From their data also, it is apparent that very little protein is present in the terminal culture medium. Tuberculin is known to average only 0.180 to 0.300 gram per 100 cc. according to Wells and Long (1932). However, if for purposes of computation

all the total nitrogen in flask 2, table 4, is considered to be protein, then 0.025 gram nitrogen multiplied by the factor 6.25 would give 0.156 gram protein per 100 cc. If, as according to the egg albumin experiment in table 1, protein has 0.4 the reducing power of glycerol, the 0.156 gram would analyze as 0.06 gram "glycerol." If one makes an additional allowance for an error of 0.10 gram

TABLE 4

Human type H 37 after 192 days in modified Long's medium

STRAIN	TERMINAL pH	AMMONIA NITROGEN	TOTAL NITROGEN	"GLYCEROL"
		grams per 100 cc.	*grams per 100 cc.*	*grams per 100 cc.*
H 37	7.2	0.000	0.033	0.43
H 37	7.3	0.002	0.025	0.34
Uninoculated control 1	7.5	0.025	0.062	2.51
Uninoculated control 2	7.5	0.022	0.067	2.70
Blank				0.10

TABLE 5

Effect of protein precipitation on the residual "glycerol"

STRAIN	TERMINAL "GLYCEROL"		
	BEFORE TREATMENT	AFTER TRICHLOROACETIC ACID	AFTER TRICHLOROACETIC ACID-KAOLIN
	grams per 100 cc.	*grams per 100 cc.*	*grams per 100 cc.*
H 37	0.63	0.63	0.53
N. Tb. A. bovine	0.34	0.26	0.39
BCG	0.37	0.48	0.34
Uninoculated control 1	3.64	3.65	3.61
Uninoculated control 2	3.58	3.60	3.63

as in the blank (table 1), then there still would be 0.18 gram "glycerol" of the terminal 0.34 gram per 100 cc. to be accounted for. Consideration of such work as that of Hooper, Renfrew, and Johnson (1932) indicates that a good share of this non-nitrogenous residuum may be carbohydrate. These investigators found about 0.120 gram carbohydrate per 100 cc. culture medium. Such carbohydrate usually contains less than 1 per cent nitrogen

according to the summary presented by Wells and Long (1932) Therefore it would seem reasonable to assume that very little if any of the residual material of tables 4 and 5 has its origin in glycerol and that *M. tuberculosis* has indeed oxidized the glycerol completely to carbon dioxide and water.

RESULTS OF THE CARBOHYDRATE STUDIES

Table 6 summarizes the present results together with those of other investigators who have employed direct analytical methods

TABLE 6

Carbohydrate utilization by Mycobacterium tuberculosis

(Based on analyses for reducing sugar)

INVESTIGATOR	TYPE	GLUCOSE	MANNOSE	FRUCTOSE	GALACTOSE	ARABINOSE	XYLOSE	LACTOSE	MALTOSE	SUCROSE
Gamble and Herrick (1922)	Human	+								
	Bovine	+								
	Avian	+								
Frouin and Guillaumie (1923)	Human							?	+	
	Bovine							?	+	?
	Avian							+	?	+
Frouin and Guillaumie (1926)	Human	+								
	Bovine	+								
Henley (1929)	Human	+								
Merrill (1930)	Human	+		+	+	+		0		0
	Avian	?		+	0	+		0		+
Model (1933)	Human	+		+						
Author	Human	+	0	?	?	+	+	0	+	
	Bovine	+	+	+	+	+	+	0	+	
	BCG	+	+	+	+	+	+	0	+	

\+ = Utilization according to analysis for reducing sugar.
0 = No utilization according to analysis for reducing sugar.
? = Doubtful—at or just beyond the limit of error of the method.

in studying the carbohydrate utilization of *M. tuberculosis*. H 37 failed to grow in the Long's carbohydrate mediums as did BCG in Long's-arabinose and Long's-lactose, so in these instances the conclusions are based on the analysis of two tubes, whereas in the other sugars 3, 4, or 5 tubes were analyzed.

The action of *M. tuberculosis* upon these carbohydrates was more vigorous in the modified Long's medium than in the glycerol beef-heart infusion broth. For instance, a heavy growth of BCG in maltose broth used no maltose but an apparently equal growth in Long's-maltose medium used about one-half the available maltose. Similarly in galactose broth only a small amount of sugar was metabolized while in Long's-galactose medium the utilization was complete. For this reason it is believed that if H 37 had grown in Long's medium, definite utilization of mannose, fructose, and galactose would have been obtained. The growth of all three strains in lactose was comparable to that in the other sugar tubes even though lactose was not utilized.

TABLE 7

Percentage of available carbohydrate utilized by M. tuberculosis in 4 months

TYPE	GLUCOSE		MANNOSE		FRUCTOSE		GALACTOSE		ARABINOSE		XYLOSE		LACTOSE		MALTOSE	
	L	B	L	B	L	B	L	B	L	B	L	B	L	B	L	B
Human	–	72	–	8	–	11	–	12	–	44	–	48	–	7	–	22
Bovine	100	80	91	86	29	13*	43	31	73	48	58	48	5	0	24	22
BCG	100	38	98	39	43	8	100*	19	–	37	100	45	–	4	41	4

L = Long's modified medium; B = Glycerol beef heart infusion broth.
– = No growth.
* 1 tube.
Note: The limit of error of the method of analysis is about 10 per cent.

Table 7 indicates the degree of utilization of the carbohydrates in the two media. Glucose, mannose, arabinose, and xylose were preferred. The percentages are averages of two or three tubes unless otherwise indicated and represent the portion of available sugar which was metabolized. The terminal pH of all broth cultures was 7.6 or above as were all bovine cultures in the modified Long's medium. BCG cultures in the modified Long's medium had a terminal pH of 7.6 or above except mannose which was 7.0 and 7.1, galactose 7.3, and maltose 7.0 and 7.5.

SUMMARY

Certain aspects of the glycerol metabolism of *Mycobacterium tuberculosis* can be studied by a method of oxidation with di-

chromate in which all organic material analyzes as glycerol. This is possible, provided a synthetic medium is used in which the oxidizable matter other than glycerol is at a minimum. Long's medium and that of Model are suitable.

It was found that human, bovine, and avian tubercle bacilli utilized as much as 96 per cent of all oxidizable matter in Long's medium. This indicates that the glycerol was completely oxidized to carbon dioxide and water and that there was no appreciable accumulation of organic waste products. This in turn suggests that the decrease in pH which occurred was not caused primarily by the presence of organic acid.

Quantitative analyses for reducing sugar indicate that human, bovine, and BCG types of *Mycobacterium tuberculosis* utilize glucose, mannose, fructose, galactose, arabinose, xylose, and maltose, but not lactose.

REFERENCES

Dingle, J. H., and Weinzirl, John 1932 Jour. Bact., **23**, 281.

Frouin, A., and Guillaumie, M. 1923 Compt. Rend. Soc. Biol., **88**, 1002.

Frouin, M. A., and Guillaumie, M. 1926 Bull. Soc. Chim. Biol., **8**, 1178.

Gamble, C. J., and Herrick, M. C. 1922 Amer. Rev. Tuberc., **6**, 44.

Henley, R. R. 1929 Amer. Rev. Tuberc., **19**, 660.

Hooper, F. E., Renfrew, A. G., and Johnson, T. B. 1932 Amer. Rev. Tuberc., **26**, 179.

Koch, F. C., and McMeekin, T. L. 1924 Jour. Amer. Chem. Soc., **46**, 2066

Loebel, R. O., Shorr, E., and Richardson, H. B. 1933 Jour. Bact., **26**, 139.

Long, E. R., and Seibert, F. B. 1926 Amer. Rev. Tuberc., **13**, 393.

Merrill, M. H. 1930 Jour. Bact., **20**, 235.

Merrill, M. H. 1931 Jour. Bact., **21**, 361, 375.

Methods of Analysis of the Association of Official Agricultural Chemists, 1925. Washington, D. C.

Model, L. M., Gurjeff, J. P., and Pirogoff, A. M. 1933 Z. Klin. d. Tuberk., **82**, 474.

Model, L. M. 1933 Z. Klin. d. Tuberk., **83**, 214.

Siebert, C. 1909 Centralbl. f. Bakt., I., O., **51**, 305.

Smith, Theobald. 1910 Jour. Med. Research, **18**, 185.

Tiffeneau, M., and Marie, A. 1912 Compt. Rend. Soc. Biol., **72**, 48.

Wedum, A. G. 1936 Jour. Inf. Dis., **58**, 234.

Weinzirl, John, and Knapton, F. 1927 Amer. Rev. Tuberc., **15**, 380.

Wells, H. G., and Long, E. R. 1932 The Chemistry of Tuberculosis. The Williams & Wilkins Company, Baltimore.

THE GROWTH OF MICROÖRGANISMS ON MEDIA EXPOSED TO ULTRA-VIOLET RADIATIONS[1]

EDWARD L. PRATT

Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Massachusetts

Received for publication July 8, 1936

The purpose of the investigation reported herein was to study the effect upon certain viable microörganisms of culture media irradiated with ultra-violet rays. The specific stimulus for the study was the work of Blank and Arnold (1935). One contribution which the present paper offers lies in the degree of precision of measurement which has been attained and in specifications thereof which permit precise duplication of the work reported.

The employment of the technique of the physicist in experimental microbiology is of such comparatively recent origin that it is not surprising that early reports of the biological effects of radiations are occasionally in conflict. Thus, Browning and Russ (1917) reported that agar irradiated from a pure tungsten arc was not inferior to non-irradiated agar for supporting growth of *Staphylococcus pyogenes-aureus*, while Coblentz and Fulton (1924), on the contrary, found that irradiated agar caused inhibition of growth of subsequently inoculated *Bacillus coli-communis*. Bedford (1927) corroborated the latter finding and offered as explanation the view that hydrogen peroxide produced in the medium was the growth inhibitor.

Woodrow, Bailey, and Fulmer (1927) found that ultra-violet irradiation of sucrose, glucose, calcium gluconate, or glycerol (in quartz flasks) rendered the solutions toxic to *Saccharomyces cerevisiae*, and that the "toxic substance" was not destroyed by boiling or by standing for 7 days. Proks (1933) reported that

[1] Contribution No. 79 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

irradiation of culture media previous to inoculation renders them less suitable for supporting bacterial growth.

The Blank and Arnold (1935) report is in general confirmation of the view that ultra-violet irradiated culture media are rendered less suitable for subsequent support of bacterial growth, and emphasizes the fact that the carbohydrates in such media are involved in the production of growth inhibitors. They report also that the "toxic substance" produced from irradiation of carbohydrates is quite thermostabile.

The results obtained and reported herewith, are not in entire agreement with the findings of Blank and Arnold.

Since the completion of this study and its preparation for publication, there has appeared the note of Baumgartner (1936) which agrees with our own findings in showing that there is a change of pH in carbohydrate solution, a change which was observed and reported previously by Berthelot and Gaudechon (1912), Bierry and Ranc (1924) and by Beyersdorfer and Hess (1924). These earlier authors agree that ultra-violet irradiation of carbohydrate solutions favors the change, in general, of polysaccharides to monosaccharides to alcohols, aldehydes, ketones, and acids. Eventually CO, CO_2, H_2 and CH_4 may be liberated, and a considerable amount of formic aldehyde may be formed. The finding of Baumgartner that a considerable proportion of the acid formed by ultra-violet irradiation of carbohydrates is formic acid is, therefore, in accord with expectancy.

EXPERIMENTAL

In our work, the radiation source was a Westinghouse 30-inch quartz Sterilamp, WL-782, mounted horizontally with supports adjustable for height, housed in a wooden box with glass front, and operated from 110 volt 60 cycle a.c. through a 2500 volt transformer at 46.5 milliamperes.

Energy emitted was measured by a non-selective thermopile with L. and N. galvanometer, type HS, on a Julius suspension and critically damped with external resistance. The standard reference lamp, type K-4 from Bureau of Standards, giving 2.807 microwatts per $mm.^2$ on 90 volts at one meter, caused a

galvanometer deflection of 3.26 cm. (average of 20 alternated standard lamp and zero readings).

By trial, it was determined that with thermopile at 4.0 cm. from the center of the Sterilamp, the average galvanometer deflection was 5.80 cm., equivalent to 49.9 ergs per mm.^{2} per second.

The spectral distribution of the light from the Sterilamp was determined by quartz spectrophotometry by a method which, while not the most precise, is reproducible and serves to define and describe the characteristics of the radiations from this light source.

TABLE 1

Spectral energy distribution of the radiations from the Sterilamp at 2500 volts and 46.5 milliamperes

WAVE LENGTH	EXPOSURE FOR STANDARD DENSITY	TOTAL ENERGY EMITTED
Ångstroms	*seconds*	*per cent*
2537	1	90.89
3132	96	0.95
3663	96	0.95
4049	64	1.41
4359	16	5.8

With a small Gaertner quartz spectrograph, loaded with an Eastman 40 plate, there was first an exposure to an ordinary quartz Uviarc (to establish the positions of the mercury lines) and succeeding exposures to the sterilamp radiations for 1 second and for increasing exposure times. The plate with its several spectrograms was developed with Eastman M-Q developer for 7 minutes at 18°C.

By visual inspection through a hand lens, the exposure time necessary to produce a line of density equal to that from 1 second's exposure of line 2537 Å was estimated. Assuming that there is no serious failure of the reciprocity law in the region covered, and with the photographic emulsion used, the relative intensities of the various mercury vapor lines would vary inversely as the exposure times. Any error involved would be due to increased

relative transmission of the quartz spectrograph and increasing sensitivity of the emulsion, both at the longer wave lengths.

In table 1 are displayed the relative exposure times of the various lines recorded, and the computed relative intensities of each.

From the above data it was estimated that 91 per cent of the radiation from the Sterilamp was of wave length 2537 Å and that the combined ultra-violet comprised nearly 93 per cent of the total radiation to which the photographic emulsion specified was sensitive.

The temperature of a Petri dish containing water in the position used for irradiating the media was after 8 hours exposure to the lamp, only 1½°C. above room temperature.

Experiment 1. Ten milliliters of sterile Blank's modified agar (Blank and Arnold, 1935) were aseptically added to each 10 cm. sterile Petri dish, allowed to harden, and then exposed with cover removed, under the Sterilamp, in such a way that the horizontal axis of the lamp was 4.0 cm. (± 1 mm.) above the agar surface. At intervals all plates were rotated, and after exposures of 3 to 6 hours were covered and removed. Control plates remained covered outside the lamp box for the same periods of time.

The inoculum was a stock culture of *Bacillus subtilis* grown for 5 days on a standard nutrient agar slant at 37°C. The growth was washed off with about 10 ml. of sterile 0.9 per cent saline, heated for 1 hour at 70°C., and the resulting suspension, regarded as a spore preparation, was swabbed onto the agar surfaces. The plates were inverted, and held at 37°C. for 72 hours.

The results, summarized in table 2, indicate marked reduction in development of spores on agar previously irradiated for 6 hours, but not always complete inhibition, and only partial inhibition from 4 hours' irradiation.

Experiment 2. All conditions were the same as for Experiment 1, except that the *B. subtilis* growth was not heated, and was a mixture of spores and vegetative cells, and the time of exposure to ultra-violet was extended. Apparently vegetative cells were less affected than spores.

Experiment 3. Difco Nutrient Agar of pH = 6.8 after sterilization was used. Inoculations were by *Bacillus subtilis* spores, or *Staphylococcus aureus*, or *Escherichia coli*. Exposures of 6 or 7 hours were employed. On this medium, *B. subtilis* spores and *S. aureus* vegetative forms were inhibited from developing; *E. coli* growth was only partly inhibited.

Experiments 1, 2, 3 are summarized in table 2.

TABLE 2

Summary of results in Experiments 1, 2, and 3

EXPERIMENT	EXPOSURE	INOCULUM	GROWTH ON EXPOSED PLATES	GROWTH ON CONTROL PLATES
	hours			
1	3	*B. subtilis* spores	3+, 4+	4+
	4	*B. subtilis* spores	3+, 3+	4+
	5	*B. subtilis* spores	2+	4+, 4+
	6	*B. subtilis* spores	−, − , ±	4+, 4+, 4+
			−, 2+	4+, 4+
2	6	*B. subtilis* vegetative cells and spores	2+	4+
	8	*B. subtilis* vegetative cells and spores	1+, 1+	4+, 4+
3	6	*B. subtilis* spores	−	4+, 3+
	7	*B. subtilis* spores	−, −, −	4+, 4+
	6	*S. aureus*	−	4+
	7	*S. aureus*	−, −, −	4+, 4+
	6	*E. coli*	1+, 1+	3+, 4+
	7	*E. coli*	1+, 2+	4+, 4+

Key: 4+ = density of growth on majority of control plates; 3+ = about 75 per cent growth density of controls; 2+ = about 50 per cent growth density of controls; 1+ = about 25 per cent growth density of controls; ± = a few scattered colonies; − = no macroscopically visible growth.

Experiment 4. The observation of yeast and mold growth on previously irradiated plates led to a series of experiments to determine if Malt Extract Agar would be improved as a medium for isolation of yeasts and molds by such treatment.

Difco Malt Extract Agar was irradiated in Petri dishes for 8 hours. Inoculation with the juice of 3 unwashed, native Baldwin apples pressed into distilled water, made up to 1 pint, diluted

1:9 or 1:99 was carried out by the method of Stuart (1935). Inoculated dishes stood for 1 hour, covered, before irradiation commenced. Subsequent incubation at 23.5 to 25.0°C. showed no superiority over unirradiated controls.

The results with grape juice from half a pound of South American white grapes showed likewise no advantage for irradiated malt agar. Pure cultures of yeast also were larger and more numerous on untreated agar. Certain motile spore-forming bacteria isolated from grape juice were inhibited, however, by the irradiated malt agar.

The treated medium is somewhat more favorable for development of molds.

In view of the suggestion of Baumgartner (1936) concerning acid production through irradiation it is important that this aspect of change in irradiated media as affecting mold growth be confirmed.

Experiment 5. A repetition of the work of Blank and Arnold (1935) in respect to irradiation of carbohydrate solutions failed to confirm these findings completely. One per cent glucose solutions were changed by 2-hour irradiation from pH 5.8 to 5.2, and after 9 hours to less than 5.0.

The agar plates made up with such irradiated solutions had a pH of 6.0 as against 6.2 for control media. Irradiation of sugar solution up to 6 hours had no effect on subsequent growth; from 8 to 12 hours of irradiation resulted in increasing inhibition of growth of *B. subtilis* spores, but no complete inhibition was reached.

A test upon 1 per cent glucose solutions buffered with Na_2HPO_4 to pH 7.8 and irradiated for 8 hours, showed about the same partial inhibition as for irradiated, unbuffered solution of original pH 5.8. If Baumgartner is correct in asserting that pH change is the factor of inhibition, these results are out of line with expectancy.

SUMMARY AND CONCLUSIONS

Six hours of ultra-violet irradiation of 10 cc. portions of agar medium of Blank's formula, or of Difco Nutrient Agar, or of

Difco Malt Agar, at about 50 ergs per mm.^{2} per second with light of which 90 per cent was of wave length 2537 Å., rendered the agar less suitable for the development of subsequently inoculated *Bacillus subtilis* spores, and also, but to a less degree, for the development of certain vegetative forms of bacteria.

The same period of irradiation of 1 per cent glucose solutions had no significant effect. Irradiation of carbohydrates in solution produced the expected shift in pH toward greater acidity.

The differences in amount of pH shift in this work and in the work of Baumgartner, and the difference in inhibitory effect found by us and that found by Blank and Arnold suggest that conditions in the three sets of investigations were probably not identical.

It is important in research involving the applications of radiant energy to chemical systems, that sufficient detail in energy identifications and measurements be reported to enable exact repetition of technic by others.

REFERENCES

Anderson and Stuart 1935 Jour. Bact., **30**, 207.

Baumgartner, J. G. 1936 Jour. Bact., **32**, 75.

Bedford 1927 Brit. Jour. Exp. Path., **8**, 437.

Berthelot and Gaudechon 1912 Compt. rend., **155**, 401.

Beyersdorfer, P., and Hess, W. 1924 Ber., **57B**, 1708; from Chem. Abst., **19**, 472.

Bierry and Ranc 1924 Bull. soc. chim., **35**, 771.

Blank, I. H., and Arnold, W. 1935 Jour. Bact., **30**, 507.

Browning, C. H., and Russ, S. 1917 Proc. Roy. Soc., Series B, **90**, 623.

Coblentz, W. W., and Fulton, H. R. 1924 Scientific Papers of Bureau of Standards, **19**, 641.

Harrison, G. R. 1929 Jour. Opt. Soc. Amer., **19**, 267.

McLeod and Gordon 1923 Jour. Path. and Bact., **26**, 326.

Pratt, E. L. 1936 Bachelor of Science thesis, Massachusetts Institute of Technology.

Proks, J. 1933 Lait, **13**, 331; from Chem. Abst., **27**, 3233.

Woodrow, J. W., Bailey, A. C., and Fulmer, C. A. 1927 Plant Physiol., **2**, 171.

LYTIC ACTION OF CERTAIN STRAINS OF HEMOLYTIC STREPTOCOCCI ON FRESH STERILE KIDNEY AND OTHER TISSUES

BEATRICE CARRIER SEEGAL AND DAVID SEEGAL

Departments of Bacteriology and Medicine, College of Physicians and Surgeons, Columbia University and The Presbyterian Hospital, New York

Received for publication July 10, 1936

During a study of the biochemical and immunological characteristics of a group of hemolytic streptococci recovered from throat cultures taken in New Orleans and New York (Teiger and Seegal, 1936), a delicate test for the proteolytic action of these organisms was employed. The kidneys from exsanguinated monkeys[1] were removed aseptically and cut into pieces 0.5 to 1 cm. square by 1 to 2 mm. thick. These were dropped into tubes of meat infusion broth, at pH 7.6, and incubated 24 hours to test for sterility. The tubes then were innoculated with 94 of the strains of hemolytic streptococci obtained in New Orleans and New York and were placed in the incubator at 37.5°C. for 3, 4, or 5 days, together with uninoculated control tubes. At the end of the incubation period the cultures were streaked on blood plates to establish their growth and purity, the pH of the broth was tested, and the pieces of monkey kidney were fixed in Zenker's, sectioned and stained with hematoxylin and eosin.

Histological examination of the sections of kidney grown with the streptococci showed that certain strains of hemolytic streptococci exert an intensely lytic action on monkey kidney tissue. The pieces of kidney taken from cultures of such strains showed destruction of nuclei and cell boundaries with loss of cellular material. Frequently the lysis was so complete that only the

[1] These monkeys were obtained through the courtesy of Dr. C. W. Jungeblut. Most of the animals were paralyzed from experimental poliomyelitis.

connective tissue frame work of the kidney remained to identify the organ. The glomerular and tubular cells were reduced to wraiths of poorly staining amorphous material without cell boundaries or nuclei. The pieces of kidney from tubes inoculated with nonproteolytic hemolytic streptococci and from the tubes of uninoculated broth appeared normal except for minimal autolytic changes. These findings are shown in figures 1 and 2. Figure 1 illustrates a piece of monkey kidney incubated for four days with a nonlytic hemolytic streptococcus and shows only minimal autolytic changes. Figure 2 illustrates a section of monkey kidney incubated for four days with a nephrolytic hemolytic streptococcus which has destroyed cell boundaries and nuclei and most of the cell cytoplasm.

Forty of all the strains tested were lytic for monkey kidney. These strains gave β-hemolysis in rabbit-blood pour plates (Brown, 1919), and belonged to Lancefield's Group A (1933). Fifty-four of the strains failed to lyse monkey kidney tissue. Forty-eight of these were not β-hemolytic streptococci and did not belong to Group A; four other strains were β-streptococci but did not belong to Group A; the remaining 2 strains were β-hemolytic streptococci and did belong to Group A. These latter two hemolytic streptococci were therefore culturally and immunologically similar to the nephrolytic strains but on two testings failed to show nephrolysin. It is possible that prolonged cultivation on artificial media may result in a loss of nephrolytic activity which proceeds faster with some organisms than with others. Among the nephrolytic strains there was apparently a difference in the amount of nephrolysin produced since some organisms produced more complete destruction of the kidney tissue than others. A study of the factors which may influence nephrolysin production is in progress.

CONTROL TESTS

A number of control tests were run in order to rule out a nonspecific cause for the lysis of the kidney tissue. They may be summarized as follows:

1. The final pH of the broth containing monkey kidney did

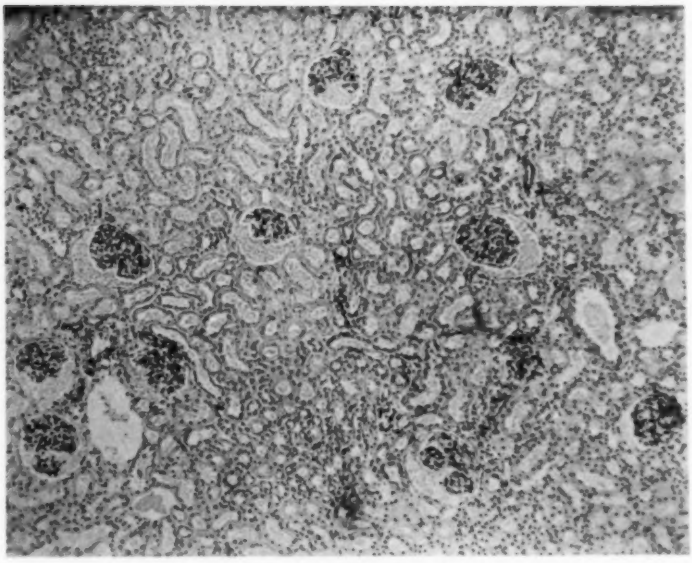


FIG. 1. SECTION OF MONKEY KIDNEY INCUBATED FOUR DAYS WITH A HEMOLYTIC STREPTOCOCCUS WHICH HAD NO NEPHROLYTIC ACTIVITY

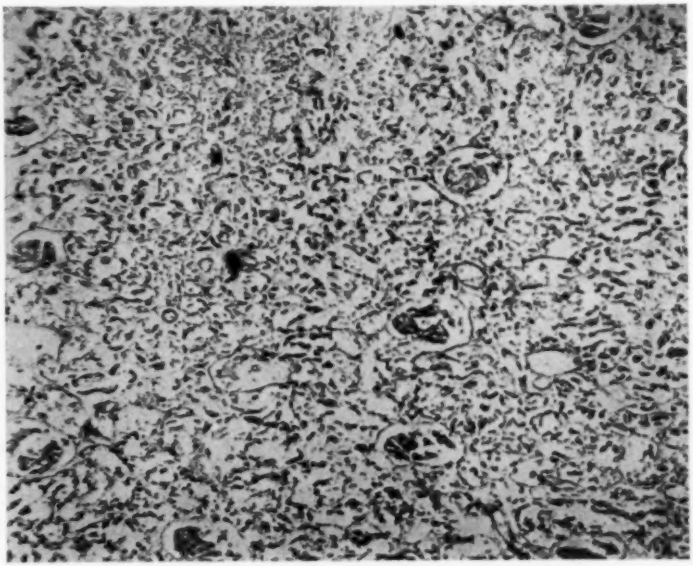


FIG. 2. SECTION OF MONKEY KIDNEY INCUBATED FOUR DAYS WITH A NEPHROLYTIC HEMOLYTIC STREPTOCOCCUS

not vary appreciably irrespective of the strains of streptococci grown. It lay between 6.7 and 7.0. The results of the pH determinations indicate that the lytic effect is not due to the final pH of the culture.

2. The effect exerted by the type of media on the lysis of the kidney tissue was tested by growing 11 selected strains in a variety of media containing the kidney tissue. Nine of the strains were known to lyse monkey kidney, while two did not. The broths employed were beef-heart infusion broth with either 1 per cent bacto- or neo-peptone, beef-muscle infusion broth with either 1 per cent bacto- or 2 per cent proteose peptone, Difco veal broth with 2 per cent proteose peptone, and the supernatant fluid from the chopped beef-heart broth used in Frobisher's histase test (1926) to be described later. No peptone was added to this latter media.

The nephrolytic strains of organisms lysed the monkey kidney in all the media tested. However, the lytic streptococci grown in the peptone free supernantant fluid from Frobisher's chopped beef-heart broth gave the most complete lysis. The nonlytic organisms did not become lytic in this peptone-free medium. This was substantiated by growing twenty-three additional known nonlytic strains with monkey kidney in this medium.

During the course of the experiments on the effects of nephrolysin which have extended over a period of a year and a half, three lots of broth have been encountered in which only slight lysis of kidney tissue occurred. These were 2 lots of beef-muscle infusion broths with proteose peptone added and one supernatant fluid from chopped beef heart without peptone. The reason for these unsatisfactory results has not been determined but probably depends upon a variation in the broth rather than a change in the microörganisms which have proved maximally lytic on subsequent testing in new media.

3. The pieces of monkey kidney varied considerably in size but pieces larger than the average lysed as readily as pieces smaller than the average. The nephrolytic substance was apparently diffusible through the larger pieces of kidney. This was further substantiated by the observation that serial sections

of four pieces of kidney showed practically the same degree of lysis throughout

4. One day's growth was insufficient to demonstrate the lytic effect satisfactorily. At the end of two days, lysis was marked and at the end of three days of growth, practically a maximum of lysis was obtained.

5. Twenty strains of the hemolytic streptococci were grown anaerobically as well as aerobically in the kidney broth. Although all grew anaerobically, the lytic effect was less pronounced anaerobically than aerobically with the exception of the case of one strain which was actively lytic when grown anaerobically, although it was not lytic when grown aerobically. This strain was not a Group A organism nor did it produce β-hemolysis in pour plates.

6. Twelve selected strains of nephrolytic and non-nephrolytic hemolytic streptococci were tested for their lysis of other monkey organs. The nephrolytic strains partially lysed monkey skeletal muscle, heart muscle, spleen, and liver. The muscle cells were more resistant to lysis than the kidney tissue. The control uninoculated sections of spleen and liver showed considerable autolytic disintegration which made the evaluation of the additional lytic effect of the streptococci difficult.

7. The same twelve strains used above were tested for their lysis of kidney tissue from other species of animals. Those strains of hemolytic streptococci which lysed monkey tissue also lysed sections of kidney from the rat, rabbit, guinea pig, and dog. The monkey kidney was the most satisfactory to use, however, since the control sections of this kidney showed only a minor degree of autolysis, even after five days incubation, whereas the kidneys from the other species showed considerable autolysis in the control tubes, rendering the evaluation of the lytic effect of the streptococci more difficult. However, the lytic effect was not species specific.

8. Representative cultures of lytic and nonlytic hemolytic streptococci were planted in broth containing monkey kidney which had been heated at 100°C. for ten minutes. The purpose

of this experiment was to destroy the autolytic enzymes of the kidney tissue in order to rule out the possibility that the lytic effect of the streptococci was merely an enhancement of the action of the proteolytic enzymes already present in the fresh kidney tissue. If lysis by the streptococci were still produced, it might be assumed that this lysis was accomplished by a product of the streptococcus acting directly on the kidney cells and not through an enhancement of the autolytic enzymes of the kidney tissue. The experiments indicated that the lytic effect of the streptococci was independent of autolytic enzymes in the kidney tissue.

9. Filtrates of hemolytic streptococci were tested for the nephrolytic agent with the following results:

a. Filtrates from cultures grown for 24 or 48 hours were slightly lytic when incubated for three or five days with monkey kidney tissue. The addition of kidney tissue to the broth from which the filtrate was obtained did not alter the results.

b. The lytic effect was completely destroyed by heating for two hours at 100°C

A concentration of the lytic activity of the filtrates is essential for further studies with this sterile substance.

A COMPARISON OF THE ABILITY OF STRAINS OF HEMOLYTIC STREPTOCOCCI TO PRODUCE A NEPHROLYSIN, TO LYSE CHOPPED MEAT, TO PRODUCE A SOLUBLE HEMOLYSIN AND TO PRODUCE A FIBRINOLYSIN

The lytic action of hemolytic streptococci on monkey kidney is a relatively delicate reaction since it is impossible to be certain of lysis without histological study. In this respect the reaction apparently differs from the "histase" reaction described by Frobisher (1926) which consists in measuring the amount by which the level of meat in a chopped meat broth tube has been reduced following incubation with a hemolytic streptococcus. The difference may be a quantitative rather than a qualitative one, however. The possible relation of the lytic action on monkey kidney to the fibrinolytic action of hemolytic streptococci described by Tillett and Garner (1933) is also interesting. The nephrolysin appears to be less species specific than the fibrino-

lysin, although Yen (1935) has reported lysis of rabbit as well as human clot with a concentrated preparation of hemolytic streptococcus fibrinolysin.

Fifty-nine strains of hemolytic streptococci from New Orleans and New York were tested simultaneously for their production of a soluble nephrolysin, hemolysin, fibrinolysin, and their ability to lyse chopped meat. The results of the tests are given in table 1. The hemolysin was tested for by the method given in the preceding paper (Teiger and Seegal, 1936). The fibrinolysin was tested in oxalated human plasma by the method of

TABLE 1

A comparison of hemolysis, fibrinolysis, "histase" reaction, and nephrolysis by hemolytic streptococci

NUMBER OF STRAINS	POUR PLATE β-HEMOLYSIS	LANCEFIELD'S GROUP A	RABBIT CELL, HEMOLYSIS 2 HOURS	FIBRINOLYSIS, TIME FOR LYSIS	HISTASE REACTION MM. LYSIS	NEPHROLYSIS
29	+	+	+	13 minutes to 1 hour 45 minutes*	5–14	+
1	+	+	+	23 minutes	2	0
1	+	0	+	36 minutes	3	0
3	+	0	+	None to 50 per cent, 24 hours	0–5	0
20	0	0	0†	None, 24 hours	0–2	0
5	M	0	Trace to complete	None, 24 hours	0–4	0

* One organism required 2 hours and 40 minutes.

† One organism showed 50 per cent hemolysis in 2 hours.

M = minute streptococci of Bliss and Long (1934).

Tillett and Garner (1933). The histase reaction was carried out in tubes of chopped beef-heart media incubated aerobically. The degree of lysis as indicated by the reduction in the height of the chopped meat was read after five days incubation and 28 days at room temperature.

It will be seen from the table that a certain correlation of hemolysin, fibrinolysin, histase reaction, and nephrolysin exists in this small group of organisms. Twenty-nine strains of Group A streptococci produced a soluble hemolysin, fibrinolysin, and nephrolysin, and were actively lytic for chopped beef heart.

Another five strains, only one of which was a Group A, failed to show any nephrolytic effect, gave little or no lysis of chopped meat, and were irregular in the production of a soluble fibrinolysin but still produced sufficient soluble hemolysin to lake the test amount of rabbit red blood cells. The remaining 25 strains were relatively inactive in all the lytic tests except for an irregular amount of soluble hemolysin production by the minute streptococci of Long and Bliss.

It would appear from these results that only hemolytic streptococci which produce a soluble hemolysin and fibrinolysin and which give Frobisher's "histase reaction" also produce a nephrolysin. On the other hand, a few strains of hemolytic streptococci which produced a soluble hemolysin and fibrinolysin and had a slight histase reaction, failed to produce a nephrolysin. This may be due to a different mechanism involved in these reactions or to a quantitative difference in the amount of proteolytic substance required for a given reaction. An attempt to answer this question by the use of sterile filtrates is now in progress.

CONCLUSIONS

1. Ninety-four strains of hemolytic streptococci were grown in broth to which pieces of fresh, sterile monkey kidney had been added. Forty of these 94 strains lysed the kidney tissue.

2. The streptococci of this limited series which produced this nephrolysin were β-streptococci of Lancefield's Group A.

3. The nephrolytic effect exerted by these 40 strains was neither organ nor species specific. The same strains of hemolytic streptococci which produced a nephrolysin lysed monkey skeletal and heart muscle, spleen and liver, and the kidney tissue of the rat, rabbit, guinea pig, and dog.

4. However, the monkey kidney tissue proved the most satisfactory test organ of the lytic effect since only slight autolysis occurred in the control sections.

5. Sterile filtrates of nephrolytic hemolytic streptococci produced slight lysis of the tissue when incubated with pieces of monkey kidney.

6. Fifty-nine strains of hemolytic streptococci were tested simultaneously for their production of a nephrolysin, a soluble hemolysin, a fibrinolysin, and a "histase" enzyme (Frobisher). Twenty-nine of these strains produced all four of these lytic effects and 20 of the strains failed to produce any of them. The remaining 10 strains failed to produce any nephrolysin but produced one or more of the other lytic reactions.

REFERENCES

BLISS, E. A., AND LONG, P. H. 1934 Jour. Bact., **37**, 105.
BROWN, J. H. 1919 Mono. Rockefeller Inst. Med. Res. No. 9.
FROBISHER, M., JR. 1926 Jour. Exper. Med., **44**, 777.
LANCEFIELD, R. 1933 Jour. Exper. Med., **57**, 571.
TEIGER, P., AND SEEGAL, B. C.
TILLETT, W. S., AND GARNER, R. L. 1933 Jour. Exper. Med., **58**, 485.
YEN, A. C. H. 1935 Proc. Soc. Exper. Biol. and Med., **32**, 1403.

A COMPARATIVE STUDY OF HEMOLYTIC STREPTOCOCCI ISOLATED FROM THE THROATS OF RESIDENTS OF NEW ORLEANS AND NEW YORK

PAUL TEIGER AND BEATRICE C. SEEGAL

Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York

Received for publication July 10, 1936

A comparative study of the geographic distribution of rheumatic fever, scarlet fever and acute glomerulonephritis in Canada and the United States (Seegal, Seegal and Jost, 1935) has shown that two of these three diseases, rheumatic fever and scarlet fever, diminish in frequency in the South, while acute nephritis remains approximately as frequent in the South as in the North. Since clinical and laboratory data indicate that acute glomerulonephritis is usually initiated by a hemolytic streptococcus infection, it appeared peculiar that the incidence of this disease failed to diminish in the southern United States while the incidence of the other two diseases due to hemolytic streptococci did decrease.

During a further study of the incidence of acute glomerulonephritis in southern latitudes, Seegal, Seegal, Jost and Cameron (1935) obtained a series of throat cultures from cases of acute nephritis and acute pharyngitis, and from normal control throats of persons living in New Orleans, Louisiana. The strains of hemolytic streptococci isolated from these throat cultures were brought back to New York. The present report consists of a study of certain biological and immunological characteristics of 63 of these strains compared with 103 strains isolated in New York City.[1]

[1] In both New Orleans and New York a number of minute hemolytic streptococci of the type described by Bliss and Long (1934) were isolated. These organisms are omitted from this study since the cultural characteristics of several of them have already been presented by Long and Bliss (1934), and the remainder of our strains are similar to these.

SOURCE OF CULTURES

The source of the strains from New Orleans has been given previously and the technique of carrying out the throat culture has been described (Seegal, Seegal, Jost and Cameron, 1935).

The strains isolated in New York were obtained by Miss Jost, Dr. Lyttle, and Dr. D. Seegal from throat cultures which, for the most part, came from cases of acute, chronic, and healed nephritis in the Presbyterian Hospital, the Babies Hospital, and the nephritis hypertension clinic of the Vanderbilt out-patient clinic. An acute upper respiratory infection was frequently present at the time the cultures were taken.

The standard strain, Dochez NY5, was used as a control strain in the tests employed.

METHOD OF STUDY

1. Blood-agar pour plates. Fifteen-cubic-centimeter volumes of beef-infusion agar containing neopeptone were placed in test tubes and stored until needed.

A blood pour plate was prepared as follows: the stock culture was transferred to a tube of rabbit-blood broth and incubated for 18 hours. The tube was shaken and a loopful placed in a tube containing 2 cc. of broth. One loopful of this diluted culture was transferred to a tube of melted agar, cooled to 50°C., to which 0.8 cc. of defibrinated rabbit's blood had previously been added. The tube was thoroughly mixed and poured. This method usually gave a very satisfactory plate for study. The plates were incubated for 48 hours at 37°C. and for 48 hours at room temperature, and were observed at intervals of 24, 48, and 96 hours.

The streptococci were classified as either producing or failing to produce β-hemolysis in the pour plates. Beta hemolysis, according to Brown's (1919) classification, consisted of a zone of perfectly clear hemolysis immediately surrounding the deep colonies, and containing no unhemolyzed red blood cells. The great majority of the β-hemolytic streptococci in this series also proved to be members of Lancefield's Group A. Colonies of Group A organisms in pour plates produced a zone of hemolysis

which varied from 2 to 3 mm. in diameter. This is in contrast to the wider zones of β-hemolysis which have been found in pour plates of organisms of Groups C and G (Seegal, Heller and Jablonowitz, 1936).

The organisms which failed to produce β-hemolysis in pour plates were either the α′ streptococci of Brown, the deep colonies of which are surrounded by an inner zone of unhemolyzed cells and an outer zone of hemolysis; or they were organisms, the deep colonies of which were surrounded by a hazy zone of hemolysis containing unhemolyzed cells throughout.

2. Hemolysis of washed rabbit red blood cells. A modification of McLeod's method (1911) was used to determine the production of a soluble hemolysin. The cultures were grown for 12 to 14 hours in 20 per cent horse serum broth. One-half-cubic centimeter quantities of the centrifuged supernatant of these cultures were added to an equal volume of a 2 per cent suspension of washed rabbit red blood corpuscles and incubated at 37°C. The final reading was taken at the end of two hours. The usual control of uninoculated broth and cells was included.

3. Fermentation reactions. Tubes of broth containing 1 per cent of lactose, mannitol, and salicin were inoculated with the cultures, incubated for 48 hours, and tested for acidity, using brom-cresol purple as an indicator.

4. Hydrolysis of sodium hippurate. The tests were carried out according to the directions of Ayers and Rupp (1922). Tubes of 1 per cent sodium hippurate broth were inoculated with the cultures and incubated for 96 hours. Ferric chloride solution (12 per cent ferric chloride + 2.5 cc. hydrochloric acid per liter) was added to 1 cc. of the clear supernatant culture fluid. A positive reaction consisted of a heavy precipitate. The amount of ferric chloride solution added was that which would insure complete clearing in an uninoculated control tube.

5. Final pH in glucose broth. According to the method of Avery and Cullen (1919), 1 per cent glucose broth was inoculated with the cultures and allowed to incubate for 96 hours. Four cubic centimeters of distilled water and one drop of methyl red were added to 1 cc. of the centrifuged supernatant. The pH

was determined colorimetrically by comparing with known standards.

6. Production of a soluble skin-toxin. Flasks of proteose peptone broth prepared according to Wadsworth's directions (1927) were inoculated from seed tubes, incubated for 48 hours, and filtered through a Berkefeld candle.

Before testing, the filtrate was diluted 1:50 and 1:100. A portion of the dilution 1:50 was heated for 2 hours at 100°C. The three different portions were then tested in the skins of chinchilla or silver fox rabbits by the injection of 0.1 cc. of each intracutaneously. The reactions were read after 24 hours. All tests were made on at least two animals, the reactivity of which to 1 STD of Dick toxin had been proved.

A reaction was called positive when the heated toxin gave either a negative or very slight reaction while the unheated portion gave a marked area of erythema 1 cm. in diameter or more.

7. Grouping. Serum against a Group A strain was prepared according to the method of Lancefield (1933). With this serum a precipitin test was performed with the hydrochloric acid extracts of the various organisms.

A slight variation of the technique has been used, however. Extracts of the growth of 100 cc. of broth have been found sufficient in almost every case. In order to save serum, the tests were carried out in small precipitin tubes of 3 mm. diameter.

RESULTS

In table 1 the results of the biochemical and serological tests are shown. These results may be most concisely described if the organisms are divided into those which produced β-hemolysis in pour plates and those which did not.

1. Beta hemolytic streptococci. The 28 β-hemolytic streptococci from New Orleans and the 54 β-hemolytic streptococci from New York proved to have practically the same cultural and immunological characteristics.

a. Twenty-six of the 28 New Orleans strains and 46 of the 54 New York strains belonged, by their fermentation of lactose,

mannitol, and salicin, to the *Streptococcus pyogenes* group (Holman (1916)).

TABLE 1

Cultural characteristics and serological grouping of hemolytic streptococci isolated from throat cultures taken in New Orleans and New York

NAME: HOLMAN'S CLASSIFICATION	NUMBER OF STRAINS	SUGAR FERMENTATION			POUR PLATE β-HEMOLYSIS	SOLUBLE HEMOLYSIN	GROUP A, LANCEFIELD	TOXIN	pH 1 PER CENT GLUCOSE BROTH	HYDROLYSIS SODIUM HIPPURATE
		Lactose	Mannitol	Salicin						
New Orleans strains										
Streptococcus pyogenes	21	+	−	+	+	+	+	+	5.0–5.4	−
	3	+	−	+	+	+	+	−	5.0–5.4	−
	1	+	−	+	+	+	−	+	4.8	−
	1	+	−	+	+	+	−	−	4.4	−
	10	+	−	+	−	−	−	−	4.4–5.2	V
Streptococcus anginosus	2	+	−	−	−	−	−	−	5.2	−
Streptococcus subacidus	11	−	−	−	−	−	−	−	4.8–5.4	−
Streptococcus hemolyticus III	10	−	+	−	−	−	−	−	5.2	−
Streptococcus equi	2	−	−	+	+	+	−	−	5.2	−
	2	−	−	+	−	−	−	−	4.8–5.2	−
New York strains										
Streptococcus pyogenes	34	+	−	+	+	+	+	+	5.0–5.4	−
	10	+	−	+	+	+	+	−	5.0–5.4	−
	2	+	−	+	+	+	−	−	4.8	−
	20	+	−	+	−	−	−	−	4.4–5.2	−
Streptococcus infrequens	1	+	+	+	+	+	+	+	5.2	−
	3	+	+	+	+	+	+	−	4.8–5.2	−
Streptococcus anginosus	9	+	−	−	−	−	−	−	4.8–5.2	−
Streptococcus subacidus	13	−	−	−	−	−	−	−	4.8–5.4	V
Streptococcus hemolyticus III	1	−	+	−	−	−	−	+	5.4	−
	6	−	+	−	−	−	−	−	5.2	−
Streptococcus equi	3	−	−	+	+	+	+	+	4.8–5.2	−
	1	−	−	+	+	+	−	−	5.2	−

V = variable.

b. Both the New Orleans and New York strains produced a soluble hemolysin active against rabbit red blood cells.

c. Twenty-four of the 28 New Orleans strains and 51 of the

54 New York strains belonged to Lancefield's Group A. This Group A was found only among the *Streptococcus pyogenes* organisms from New Orleans, but occurred also in the *Streptococcus infrequens* and *Streptococcus equi* groups in New York. The former group was not represented among the New Orleans strains. All the four *Streptococcus infrequens* organisms were of Group A, and three of the four *Streptococcus equi* from New York were also of Group A.

d. Twenty-two of the 28 New Orleans strains and 38 of the 54 New York strains produced a soluble skin toxin active in the skin of rabbits.

e. Both the New Orleans and New York strains reached a final pH in glucose broth which varied between 5.0 and 5.4 (two exceptions of pH 4.8).

f. Both the New Orleans and New York strains failed to hydrolyze sodium hippurate.

2. Hemolytic streptococci which did not give β-hemolysis in pour plates. The 35 hemolytic streptococci from New Orleans and the 49 hemolytic streptococci from New York which failed to give β-hemolysis in pour plates proved to have practically the same cultural characteristics.

a. Both the New Orleans and New York strains were distributed according to their fermentation of lactose, mannitol, and salicin in the *Streptococcus pyogenes*, *Streptococcus anginosus*, *Streptococcus subacidus*, *Streptococcus hemolyticus III*, and *Streptococcus equi* groups.

b. None of either the New York or New Orleans strains produced a soluble hemolysin active against rabbit red blood cells, belonged to Lancefield's Group A, or produced a soluble skin toxin active in the skin of rabbits (with one exception).

c. Both the New Orleans and New York strains reached a final pH in glucose broth which varied between 4.4 and 5.4.

d. Occasional strains from New Orleans and New York hydrolyzed sodium hippurate.

From these tests it is apparent that no difference between the hemolytic streptococci isolated in New Orleans and New York has been demonstrated.

SUMMARY

Sixty-three strains of hemolytic streptococci isolated from throat cultures taken in New Orleans were compared with 103 strains of hemolytic streptococci isolated from throat cultures taken in New York. The New Orleans strains came from cases of acute nephritis and acute upper respiratory infection and from normal throats. The New York strains came from cases of acute nephritis and from cases of chronic nephritis or healed nephritis, with or without acute pharyngitis.

The organisms were examined for their fermentation of lactose, mannitol, and salicin, their hemolysis in blood pour plates, their production of a soluble hemolysin and skin toxin, their hydrolysis of sodium hippurate and their final pH in glucose broth. Their membership in Group A of Lancefield also was determined.

By these tests no difference was found between the hemolytic streptococci isolated from the throats of individuals resident in New Orleans and New York.

REFERENCES

AVERY, O. T., AND CULLEN, G. E. 1919 Jour. Exper. Med., **29**, 215.

AYERS, S. H., AND RUPP, P. 1922 Jour. Inf. Dis., **30**, 388.

BLISS, E. A., AND LONG, P. H. 1934 Jour. Bact., **37**, 105.

BROWN, J. H. 1919 Mono. Rockefeller Institute Med. Res., 9.

HOLMAN, W. L. 1916 Jour. Med. Res., **34**, 377.

LANCEFIELD, R. 1933 Jour. Exper. Med., **57**, 571.

LONG, P. H., AND BLISS, E. A. 1934 Jour. Exper. Med., **60**, 619.

MCLEOD, J. W. 1911 Jour. Path. and Bact., **16** 321.

SEEGAL, B. C., HELLER, G., AND JABLONOWITZ, J. 1936. Proc. Soc. Exper. Biol. and Med., **34**, 812.

SEEGAL, B. C., SEEGAL, D., JOST, E. L., AND CAMERON, P. 1935 Proc. Soc. Exper. Biol. and Med., **32**, 1151.

SEEGAL, D., SEEGAL, B. C., AND JOST, E. L. 1935 Amer. Jour. Med. Sci., **190**, 383.

WADSWORTH, A. B. 1927 Standard Methods of Division of Laboratories and Research of the New York State Department of Health, New York. Williams & Wilkins, Baltimore.

THE PREPARATION OF SILICIC ACID JELLIES FOR BACTERIOLOGICAL MEDIA

JOHN H. HANKS AND ROBERT L. WEINTRAUB

Department of Bacteriology, School of Medicine, and Department of Botany, The George Washington University, Washington, D. C.

Received for publication July 17, 1936

Although silicic acid jellies are inferior to agar, gelatin, blood serum or egg white in the preparation of solid media for the majority of bacteriological requirements, there are certain specialized purposes, such as the isolation of autotrophic bacteria, for which an inorganic jelly is necessary. Theoretical advantages from the utilization of silica gel as a specialized substitute for organic gels have not been realized in practice, largely as a result of the erroneous assumption that inorganic gels are inert in the medium. The present study deals with the influence of time, temperature, dialysis and autoclaving on the pH and the rigidity of silica jellies prepared for bacteriological purposes.

Many modifications of two classical methods for the preparation of silicic acid jellies have been described by bacteriologists. The "sol" method, first applied in bacteriology by Kuhne (1890) and later used by Winogradsky (1891) and by Omeliansky (1899), was adequately reviewed by Bojanovsky (1925) and recently employed by Boltjes (1935) in obtaining pure cultures of nitrifying bacteria. This procedure has a distinct advantage in that the dialyzed and sterilized sol is gelled after addition of the bacterial suspensions to the plates. However, it was not considered of general utility because of the special apparatus required for the preparation of the dialyzed sol and because the liquid of syneresis exuded on the surface of the plates permits a film of contaminants to cover the pure colonies which may develop in the depths of the gel. Boltjes overcame this difficulty with the aid of a micromanipulator for colony picking.

Because of its apparently greater simplicity, the "gel-in-plate" method recommended by Winogradsky (1925) has been less carefully developed and more widely used. In this procedure, the mixture of silicate and acid, at an undetermined pH, is allowed to harden in the Petri dishes. The gel is dialyzed in tap water and distilled water to remove the excess sodium chloride, after which the nutrient salts are added. No provision is made for final sterilization. Various modifications such as those by Stevens and Temple (1908) and by Doryland (1916) have provided for sterility, but disposal of liquid of syneresis and of excess sodium chloride has been neglected. Therefore, in the case of this "gel-in-plate" method, one has been forced to choose between a good nutrient medium which could not be sterilized and a sterile medium with an unfavorable salt concentration. The production of gels which fulfill the strict requirements of uniform nutrient content, suitable pH, optimal salt concentration, dry surface, and autoclave sterility has not been accomplished by the methods hitherto available.

For several years previous to this study silica gels were prepared by the authors at pH 7.4, using bromthymol blue indicator (see methods), on the assumption that the gel should be made at the pH desired in the medium. Although the color of the medium was observed to become a deeper blue on standing, this change was attributed to an increased opacity of the gel rather than to a decreased H-ion concentration.

These gels were prepared from sterile reagents and were dialyzed aseptically in a solution of inorganic salts to impregnate the plate with nutrients and to remove the excess sodium chloride. Although such plates permitted the isolation of pure cultures of autotrophic bacteria (*Nitrosomonas*),[1] they occasionally failed to provide favorable conditions for growth. It was finally learned that these failures were due to the anomalous fact that the pH of the dialyzed gels was often higher than that of the original jelly or of the dialyzing fluid. Furthermore, the inconvenience attending aseptic dialysis was so great that this procedure was

[1] A practical method for this purpose is being published separately (Hanks and Weintraub, 1936b).

considered impracticable. The gels were next dialyzed in open baths of the nutrient solution and attempts were made to sterilize them in the autoclave. Autoclaving further increased the alkalinity of the medium and usually resulted in its complete peptization.

Therefore, it became necessary to undertake an investigation of the H-ion changes in gels made in various ways and to study the influence of dialysis and autoclaving on the rigidity and pH of the medium.

METHODS OF STUDY

The method for the preparation of the gels differed from the classical procedure. Previous titration of the reagents was made unnecessary by the addition of 5 cc. of 0.04 per cent aqueous bromthymol blue or bromcresol purple per liter of the stock solutions of sodium silicate and of hydrochloric acid. The gels were prepared at the desired pH by pouring the sodium silicate into a chosen volume of the acid in a large flask until the proper color of the indicator appeared. The pH of a small sample of the mixture was then quickly determined electrometrically and further additions of acid or silicate were made as required. In order to delay gel formation in rapidly hardening mixtures until the pH could be adjusted and the mixture could be distributed among 20 or 30 Petri dishes, the reagents were previously chilled to 4°C. All batches of gel were made in such volume that duplicate or triplicate plates (containing approximately 30 cc.) could be used for each variable studied. This method permitted the preparation of any desired number of plates in which the nutrients, the pH, the hardening time, and the SiO_2 content of the gels were standardized automatically. Furthermore, the gels were easily duplicated in successive batches.

Although firm gels were formed by mixtures of 3.5 per cent sodium silicate (Baker's 40 per cent solution, diluted to 3.5 per cent Na_2SiO_3) and 3 per cent of concentrated hydrochloric acid by volume (approximately 0.3 N), the changes in the gels during dialysis and autoclaving made it desirable to employ 6 and 5 per cent, or 7 and 6 per cent, concentrations of silicate and acid,

respectively. Since approximately equal volumes of these reagents were required, various salts were incorporated in the jellies by adding a double amount of these salts to the stock solution (acid or silicate) in which they were most stable.

All pH measurements were made at 25°C., with a calomel half-cell and quinhydrone electrode. Rapid determinations on chilled acid-silicate mixtures were made possible by keeping a number of tubes containing 5 cc. of distilled water and excess quinhydrone in a bath at 32°C. Shaking 3 cc. of the chilled mixture in one of these tubes quickly brought the temperature to 25°C. The pH of the gels was determined by macerating approximately 2 cc. of the gel with a glass stirring rod, in a short tube containing solid quinhydrone, until the gel was reduced to a smooth paste. The gel paste was then diluted with distilled water at 25°C. to a final volume of approximately 8 cc. and the suspension was shaken. Gels which had been covered with water of syneresis or with dialyzing fluid were rinsed with distilled water before samples were removed.

The gels were dialyzed at 40°C. in ten times their volume of the various solutions which were employed. Dialysis was hastened by arranging the plates, without lids, on edge in the dialyzing fluid with the exposed surface of the gels facing slightly downward.

Properly prepared gels withstood autoclaving at ten pounds of steam pressure for 15 minutes. Occasional cracks at the periphery of the medium were caused by expansion of air in this rapidly heated portion of the gel before the autoclave was sealed, but these slight imperfections did not impair the usefulness of the plates. Since bubbles and cracks throughout the medium resulted from rapid decreases in the pressure during autoclaving, the steam chamber was sealed absolutely tight after displacement of the air and the pressure was carefully controlled. Good results were also obtained by the more laborious method of raising the pressure in the tightly sealed autoclave to the desired point and then exchanging the air for steam *without permitting fluctuations in pressure.*

EXPERIMENTAL RESULTS

pH changes in non-dialyzed gels

The early observations suggesting a pH drift in gels prepared at a known pH were confirmed. Figure 1 shows the course of the alkaline shift in a batch of gel made at pH 6.3. One lot of plates was autoclaved immediately and then stored at 30°C.; a second lot was maintained at 30°C. while a third lot was kept at 4°C. Autoclaving promptly alkalinized the gel to pH 7.4. The pH

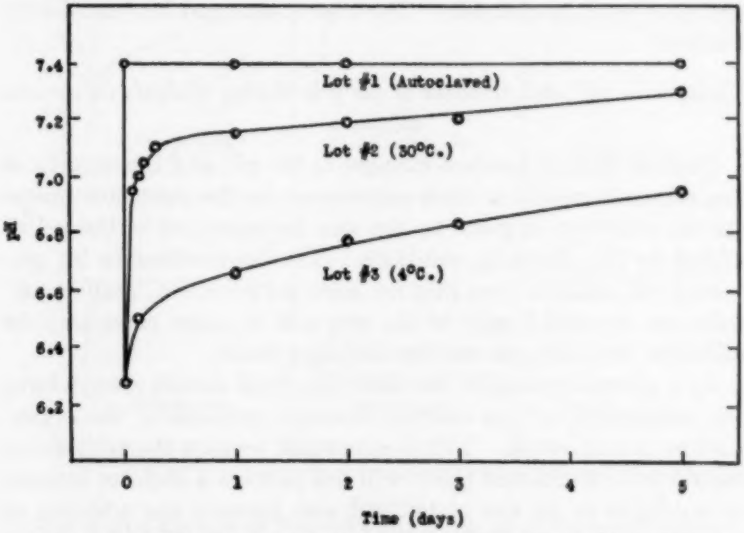


FIG. 1. THE pH CHANGES IN SILICA GELS AS INFLUENCED BY TEMPERATURE

of the gels stored at 30°C. rose rapidly at first and then more slowly; at this temperature the gels attain the stable pH of autoclaved gels after 14 to 21 days. The process of H-ion change in the jellies stored at 4°C. was much slower and more prolonged.

All batches of gel prepared between pH 6.0 and 7.5 ultimately showed a rise of 1.1 to 1.2 pH units, regardless of differences in initial H-ion concentration or in the storage temperature. These results demonstrate that the final pH of non-dialyzed jellies is much higher than the pH at which the jellies are prepared. This

phenomenon doubtless explains one of the unknown faults of the gels of Stevens and Temple, of Doryland and of those prepared earlier by the authors.

Nutrient salts can be incorporated in silica jellies at the time of preparation and the initial pH of the acid-silicate mixtures can be adjusted to produce the desired pH after autoclaving. The rise will be about 0.8 pH in non-dialyzed media which contain 0.1 per cent of phosphates. However, such gels are not generally useful for the cultivation of bacteria because of the high concentration of sodium chloride. The excess salt must be removed by dialysis.

Changes in pH and firmness of the gels during dialysis in various solutions

Dialysis induces marked changes in the pH and the rigidity of the gels and results in their peptization in the autoclave unless special attention is given to the ions incorporated in the gel or added to the dialyzing solutions. The information to be presented will make it clear that for some purposes the "stabilizing" salts can be added only to the gels and in other cases may be added to both the gels and the dialyzing fluids.

As a general principle, the dialyzing fluid should always have the composition of the nutrient solution required by the organisms to be cultivated. This is important because the addition of materials to individual plates will not provide a uniform content of nutrients in all the plates and also because the addition of substances to a finished medium may cause further changes in pH. Since nutrient solutions to be used for various purposes will differ in composition, it seemed advisable to attempt the stabilization of gels in solutions containing a simple assortment of ions. Phosphate mixture (M/150) was chosen as an ingredient which would be involved universally.

Figure 2 shows the pH of the gels and the solutions at the indicated intervals during dialysis of silica gels in various solutions, and also the pH and the condition of the gels after autoclave sterilization. It may be seen that the gels were made unsuitable for autoclaving by dialysis in distilled water of pH 5.7, in phos-

phate buffer of pH 7.4 and in phosphate buffer +0.1 per cent NaCl (pH 7.4). On the other hand, the gels were stabilized in the slightly acid phosphate buffer (pH 6.2), in the ammonium sulphate medium[2] (pH 7.4) and in the phosphate buffer +0.1 per cent NH_4Cl (pH 7.4). Comparison of the results obtained with these different solutions is instructive.

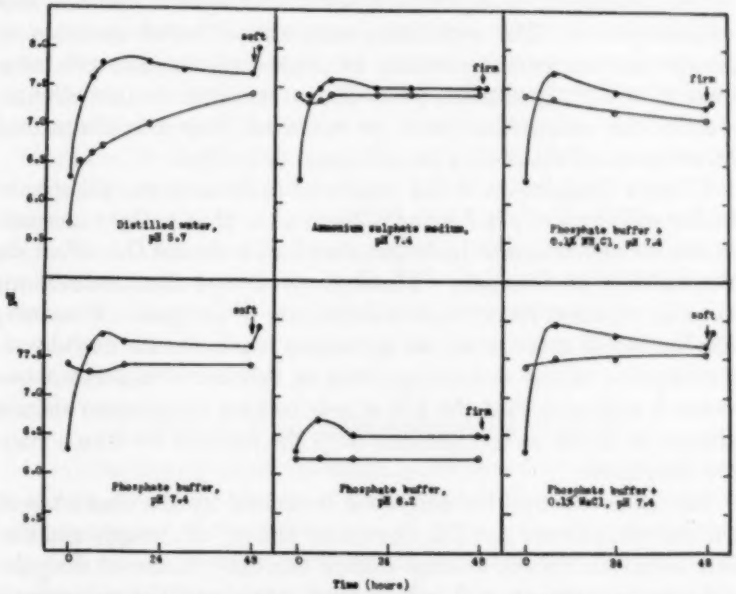


FIG. 2. THE RESULTS OF DIALYZING SILICA JELLIES IN VARIOUS SOLUTIONS
● — Gels
○ — Dialyzing fluids
↓ Arrow indicates autoclaving

In the first place it is obvious that dialysis in distilled water accomplished nothing more than the removal of excess sodium chloride and a weakening of the gel; the nutrient salts had not been introduced and the gel could not be sterilized. In contrast, dialysis in the ammonium sulphate medium produced a stable

[2] Ammonium sulphate medium: 1 gram $(NH_4)_2SO_4$, 0.2 gram KH_2PO_4, 0.8 gram K_2HPO_4, traces of $FeSO_4$ and $MnSO_4$, in 1 liter of tap water.

nutrient gel[3] which was autoclaved without appreciable change in pH or firmness. That the ammonium ion, rather than increased concentration of electrolyte, was the important factor in the stabilization of the gel at pH 7.4 is demonstrated by the fact that phosphate buffer containing 1 gram of ammonium chloride per liter also stabilized the gel, while at the same pH phosphate buffer containing an equivalent amount of sodium chloride was unsatisfactory. The stabilizing influence of small amounts of ammonium solves the problem of preparing dialyzed gels at a neutral or slightly alkaline pH. However, under certain circumstances the ammonium must be excluded from a medium and other means of stabilizing the gels must be sought.

From a comparison of the results of dialysis in the phosphate buffer solutions at pH 7.4 and 6.2 it is clear that a slight increase in the concentration of hydrogen ions had a remarkable effect on the stability of silica gels. Much less hydrogen than ammonium ion was required to produce stabilization of the gels. However, the biological activity of the hydrogen ion limits its usefulness. Recognition of the stabilizing effect of this ion is important because it indicates that the pH of gels lacking ammonium should always be as low as is consistent with the purpose for which they are prepared.

The time required for dialysis is indicated by the character of the curves in figure 2. The curves for the pH of the gels and the salt solutions always diverge during the early hours of dialysis. They then approach each other and tend to establish a constant relationship within 0.1 to 0.3 pH units. Dialysis may be regarded as sufficiently complete whenever this relationship has been attained. If dialysis is terminated before its completion, the jellies are considerably more alkaline than the dialyzing fluid and may be unstable.

Inasmuch as the non-dialyzed jellies were alkalinized during storage, it was also important to determine whether this cause of variations in the pH of stored gels would influence the final pH and firmness of the gels at the end of dialysis. Because of the

[3] Without further treatment, except drying, such gels are complete and satisfactory media for the isolation of pure cultures of ammonia-oxidizing bacteria.

temperature conditions to which they had been subjected, each lot of the jelly shown in figure 1 had a different pH at the end of 5 days of storage. The individual lots of this jelly (pH 7.4, 7.3 and 6.9, respectively) were dialyzed separately in the ammonium sulphate medium at pH 7.4. All of these gels reached identical H-ion concentrations by the end of 9 hours of dialysis. The results already described (see fig. 2) for the dialysis of freshly prepared gels in the ammonium sulphate solutions were duplicated throughout the remainder of the dialyzing period and on autoclaving. Therefore, non-dialyzed gels may be stored in any convenient way until one wishes to start the dialysis.

Since the final pH of the gels dialyzed in the buffer solution or in solutions containing ammonium is controlled by that of the dialyzing solution, some latitude in the initial pH of the gels is allowable. The gels may be reproduced with sufficient accuracy for bacteriological purposes by merely combining the acid and silicate solutions until a conveniently recognized color of the chosen indicator is observed, e.g., grass green to bromthymol blue or wine red to bromcresol purple.

The effect of various salts incorporated in the gels

On theoretical grounds, it might be expected that stabilization would be effected by the use of salts of calcium, aluminum or iron. Unfortunately, the precipitation of these ions by phosphate, which is always present, precludes their addition to the dialyzing fluids. Since it had been learned that the incorporation of the ammonium ion in the acid-silicate mixtures would protect the gel during dialysis in the plain phosphate buffer of pH 7.4, the effects of incorporating the di- and tri-valent ions in the gel were studied.

By the addition of calcium chloride, ferrous sulphate or aluminum chloride to the acid solution, jellies were made which contained the same concentration of each of the cations as was given by 1 gram of ammonium sulphate per liter. The stabilizing effects of the four cations were compared by autoclaving the gels following dialysis in the buffer solution of pH 7.4. As previously observed, the ammonium gels were stabilized and no permanent change in the pH of the dialyzing solution was induced. On the

other hand, in the calcium, iron and aluminum gels the final pH was too high for satisfactory stabilization, because of a slight alkaline drift which occurred in the dialyzing solutions. When allowance was made for this drift, by employing dialyzing solutions of pH 7.0, all the gels were of satisfactory firmness and had final hydrogen ion concentrations ranging from pH 7.2 to 7.4 after autoclaving. Of the three gels lacking ammonium, the aluminum and the iron gels were clear whereas the calcium gel became opaque during dialysis (precipitation of calcium phosphate) and did not permit microscopic examination through the bottom of the closed plate.

The gel containing ferrous sulphate, when made with bromcresol purple at an initial wine red color, quickly developed a yellow appearance and determinations with the quinhydrone electrode indicated considerable acidification. The factors involved in this apparent pH change could not be worked out because of a disagreement between the pH values obtained colorimetrically and electrometrically. However, following dialysis in the buffer solution of pH 7.0, determination with the two H-ion methods were in agreement and stable gels were produced with pH 7.2 after autoclaving. More experience with the iron gels is needed before the course of their pH changes can be adequately understood.

PREPARATION OF THE MEDIUM

The procedure finally evolved for the preparation of silicic acid media consisted of three steps:

(a) Preparation of gels in which were incorporated the salts for nutritional purposes, or for stabilizing the firmness and pH of the medium.

(b) Dialysis of the gels in solutions which removed the excess sodium chloride, provided the nutrients required in the medium, and stabilized the firmness and pH of the gel.

(c) Sterilization of the medium in the autoclave.

The precautions to be observed in autoclaving the medium have been mentioned.

For the pure-culture isolation of bacteria it is necessary that the surface of the sterilized medium be absolutely dry. The plates are covered with a towel during autoclaving. After removal of the plates from the *cooled* autoclave, each is held on edge to allow the excess fluid to drain out. The sterile towel is then touched to the crack between the plate and the lid, to act as a wick for absorbing the residual fluid, so that no capillary film remains between the halves of the dish to permit contamination. The plates are then inverted and placed at 37°C. for 48 hours or at 28°C. for 96 hours in order to dry the surface completely.

Silicic acid jellies cannot be inoculated by streaking. The inoculum can be spread evenly over the surface by holding the plate in a nearly horizontal plane and causing it to oscillate through a short arc by a rapid side to side movement of the wrist. The fluid should be taken up quickly by the gel.

DISCUSSION

The foregoing data illustrate the most obvious and, perhaps, the more important changes which take place in silica gels during the preparation of a bacteriological medium. Since the phenomenon of pH rise in non-dialyzed gels and the stabilizing influence of various ions on the pH and firmness of these gels during dialysis will be discussed elsewhere (Hanks and Weintraub, 1936a) from a more theoretical point of view, it is desirable in this paper to state only briefly the factors which are believed to be involved.

The initial pH of the acid-silicate mixtures probably represents a titration equilibrium between the hydrogen and hydroxyl ions of the acid and the silicate. The changes in pH which accompany the hardening of the jelly are explained on the basis of an ionic exchange between sodium and hydrogen ions which are competing for positions on the gel framework. The factors controlled the success with which various cations compete for positions on soils, clays, permutits and other hydrous colloids have been admirably summarized by Jenny (1932) and are known to depend on the size, charge and degree of hydration of these ions. Among the monovalent ions, the firmness of adsorption is indicated by the lyotropic series: $Na^+ < K^+ < NH_4^+ < H^+$ and the ease with

which each ion may be displaced is expressed by arranging the list in the reverse order. This means that hydrogen ions will release or displace sodium ions even though the latter are present in much greater concentration. A progressive adsorption of hydrogen ions by the gel framework and a release of sodium ions into the capillary water of the non-dialyzed gels would appear to explain the alkalinization described in this paper.

Since the pH shift in non-dialyzed jellies represents merely a redistribution of the ions, it is understood why the pH rise during the storage of plates did not influence the pH of the plates at the end of dialysis. The pH of the gel at the time it is placed in the bath is altered by further ionic exchange during dialysis. A correlation between the position of the ions in the lyotropic series and their influence on the stability of the gels was demonstrated in this study. Gels dialyzed at pH 7.4 in solutions containing only sodium or potassium ions were alkalinized and were softened or peptized on autoclaving, while equivalent concentrations of ammonium ion produced stabilization. In the absence of the ammonium ion, stabilization was secured with relatively slight increases in the concentration of hydrogen ions. These results are in agreement with the demonstration by Wiegner (1931) and Baver (1929) that the viscosity, degree of hydration and the general stability of clays and other soil colloids are markedly affected by the nature and concentration of the adsorbed ions.

The incorporation of certain salts in the gels was based on the hypothesis that properly selected ions might be adsorbed with sufficient firmness to remain in the gel during dialysis in plain buffer solution and thus promote stabilization at a slightly higher pH than was otherwise possible. From the results obtained it would appear that calcium, aluminum or iron were slightly less effective than equivalent amounts of ammonium. The disadvantages in the use of calcium and the peculiarities observed in the case of iron have been mentioned. The incorporation of more than one salt in the same gel might be advantageous.

SUMMARY AND CONCLUSIONS

1. A method, which does not require previous titration of the reagents, is given for the preparation of silica gels. This method

facilitates the preparation of large batches of jelly in which the nutrients, the hardening time, and SiO_2 content and the pH are readily duplicated.

2. The universally disappointing attempts to employ silica jelly as a solidifying agent in bacteriological media are shown to result from the fact that each step in the preparation of the medium is attended by hitherto unsuspected changes.

3. The changes which occur in the pH of non-dialyzed jellies, and in the pH and firmness of jellies during dialysis and autoclaving, are described.

4. Stabilization of the jellies during dialysis and autoclaving depends on the ions incorporated in the jelly and on the ions furnished by the dialyzing solutions.

5. Stabilization is secured by maintaining the pH below 7.0, by the incorporation of ammonium, aluminum, calcium or iron salts in the gels, or by dialysis in solutions containing the ammonium ion.

6. This study shows that it is practical to make stable, sterilizable jellies for bacteriological purposes by adherence to the following principles:

(a) Ions which tend to stabilize the gel structure during dialysis should be incorporated at the time of gel preparation.

(b) In certain instances, the ions in the nutrient dialyzing solution will afford stabilization; in such cases the incorporation of the stabilizing ion in the jelly is not mandatory but is desirable.

(c) Dialysis to remove excess sodium chloride should always be carried out in a nutrient solution of the final composition desired in the medium. The pH of the dialyzing fluid should be as low as is consistent with the purpose for which the medium is intended.

REFERENCES

BAVER, L. D. 1929 Missouri Agri. Exp. Sta. Bull. No. 129.
BOJANOVSKY, R. 1925 Centralbl. f. Bakt., II Abt., **64**, 222.
BOLTJES, T. Y. K. 1935 Arch. f. Mikrobiol., **6**, 79.
DORYLAND, C. J. T. 1916 Jour. Bact., **1**, 135.

HANKS, J. H., AND WEINTRAUB, R. L. 1936a J. Phys. Chem. (In press).
HANKS, J. H., AND WEINTRAUB, R. L. 1936b Jour. Bact., **32**, 673–687.
JENNY, HANS 1932 Jour. Phys. Chem., **36**, 2217.
KUHNE, W. 1890 Zeit. f. Biol., **27**, 172.
OMELIANSKY, V. 1899 Centralbl. f. Bakt., II Abt., **5**, 537.
STEVENS, F. L., AND TEMPLE, J. C. 1908 Centralbl. f. Bakt., II Abt., **21**, 84.
WIEGNER, G. 1931 Jour. Soc. Chem. Ind., **50**, 55, 65, 103.
WINOGRADSKY, S. 1891 Ann. de l'Inst., Past., **5**, 92.
WINOGRADSKY, S. 1925 Ann. de l'Inst. Past., **39**, 299.

THE PURE CULTURE ISOLATION OF AMMONIA-OXIDIZING BACTERIA

JOHN H. HANKS AND ROBERT L. WEINTRAUB

Department of Bacteriology, School of Medicine, and Department of Botany, The George Washington University, Washington, D. C.

Received for publication July 17, 1936

The autotrophic nature and agricultural importance of the ammonia-oxidizing bacteria has long stimulated the interest of bacteriologists. Studies of these organisms have been neglected, however, because of the difficulties in obtaining pure cultures. Such cultures were first described by Winogradsky (1890) and have since been obtained by several other investigators (Bonazzi, 1919; Heubült, 1928; Rubentschik, 1929; Nelson, 1931; Boltjes, 1935). Nevertheless, generally useful isolation methods have not been developed, as illustrated by the fact that no pure cultures were available to the writers in 1933. Since that time, Boltjes (1935) has reported an admirable study of the nitrifying bacteria and has isolated pure cultures of *Nitrosomonas*[1] and *Nitrobacter*. Inasmuch as the pertinent literature on the isolation of nitrifying bacteria has been reviewed recently by Winogradsky and Winogradsky (1933) and by Boltjes, it is necessary to point out only that Nelson's method of picking single cells is impractical, that Winogradsky (1935) in his recent work has neither sought nor possessed pure cultures and that Boltjes employed a micromanipulator for picking his colonies.

It was the purpose of the present investigation to develop a method of isolation which would require no special apparatus and in which the colonies could be picked by hand, so that the isolation could be made in bacteriological laboratories in general. The

[1] One of these cultures was obtained through the kindness of Dr. H. Humfeld of the Bureau of Chemistry and Soils, United States Department of Agriculture. This strain nitrified actively and did not grow in broth.

accomplishment of this purpose eventually necessitated a study of the preparation and behavior of silicic acid jellies. The study on silica gels is being published separately (Hanks and Weintraub, 1936) because of its possible value in connection with the use of silica jellies for other purposes. It should be consulted for detailed information concerning the silicic acid media employed in the present investigation.

In addition to the problem of a suitable plating medium, there was the difficulty of obtaining favorable ratios between *Nitrosomonas* and other organisms in suspensions to be plated. This problem arose from the predominance in soil of heterotrophic bacteria and was complicated by the fact that some of these contaminants readily outgrew the ammonia-oxidizing organism in solutions of ammonium sulphate and other nutrient salts in distilled water.

A summary of the procedure for isolation will be presented first and will be followed by more detailed information concerning the principles and the difficulties involved in the various steps.

SUMMARY OF THE ISOLATION PROCEDURE

The following stock solutions are required:

1. A concentrated solution of salts for preparing the liquid culture medium and the dialyzing fluid, and for addition to the acid solution.

$(NH_4)_2SO_4$	10 grams
K_2HPO_4	7.5 grams
KH_2PO_4	2.5 grams
$FeSO_4$	0.1 gram
$MnSO_4$	0.1 gram
Distilled water	100 cc.

This solution is diluted 1:100 in tap water to make the dialyzing fluid and the liquid medium. The liquid medium is tubed in quantities of 4.5 cc. and sterilized in the autoclave.

2. A 6 per cent solution of sodium silicate (prepared from Baker's 40 per cent solution) in distilled water, to which is added 5 cc. of 0.04 per cent aqueous bromcresol purple per liter.

3. An acid solution containing:

Concentrated salt solution No. 1	20 cc.
Distilled water	925 cc.
Concentrated HCl (37 per cent)	50 cc.
Bromcresol purple (0.04 per cent)	5 cc.

4. A sterile suspension of calcium carbonate. One gram of calcium carbonate is placed in tubes with 10 cc. of distilled water and is autoclaved. One drop of this suspension is added aseptically to each tube of the sterile liquid medium.

For the preparation of plates the sodium silicate solution is poured into a chosen volume of the acid solution in a 1-liter flask (approximately equal volumes are required) until the indicator shows the color of approximately pH 6.3. The mixture is immediately poured into Petri dishes and will harden in about 2 minutes. Batches of 25 plates, each containing about 30 cc. of gel, are usually prepared. With a little experience the pH of the mixtures can be duplicated from batch to batch with sufficient accuracy for bacteriological work. The final pH of the gel is controlled by the dialyzing fluid.

The gels may be stored in the refrigerator or placed immediately (without lids) in a quantity of dialyzing fluid which is ten times the volume of the silica gel to be dialyzed. Since tap waters in various places differ in pH, the ratio of primary and secondary phosphates in the concentrated salt solution should be chosen to give a dialyzing solution of pH 7.4. An enamel dish pan is conveniently used as a dialyzing bath. The temperature is maintained at 40°C. by a micro-burner or pilot light and evaporation is prevented by using an inverted dishpan as a cover. When dialysis is sufficiently complete (24 to 30 hours) the pH of the solution should be 7.4 and the pH of the gel should be 7.5 to 7.6. After dialysis the plates are rinsed and the lids are replaced. These gels may be stored in the refrigerator indefinitely and should not be autoclaved until just prior to the time when they are needed. The procedure for autoclaving and final drying of the medium has been given elsewhere (Hanks and Weintraub, 1936).

A dry surface on the medium is essential for pure culture isolation. Otherwise, spreading of contaminating microorganisms is inevitable.

The inoculations for pure culture isolation are made with serial dilutions of crude culture suspensions which contain suitable proportions of ammonia-oxidizing bacteria. All dilutions are prepared by transferring 0.5 cc. of the bacterial suspension from tube to tube of the liquid medium containing calcium carbonate.

The plates are inverted and incubated at 28°C. In 3 or 4 weeks the colonies usually become barely visible to the naked eye. Apparently pure colonies are picked to the liquid medium and subsequently tested for absence of growth in nutrient broth.

DETAILS CONCERNING VARIOUS STEPS IN THE PROCEDURE

The plating medium

The preparation of plating media from silicic acid jellies requires a knowledge of the pH changes which occur in the process and an appreciation of the rôle of various ions in the stabilization of the pH and the rigidity of the gels during autoclave sterilization (see Hanks and Weintraub, 1936).

It is well known that heterotrophic microorganisms will develop despite the use of recrystallized salts and redistilled water. Furthermore, tap water is preferable to distilled water for dilution of the concentrated salt solution (Stock solution No. 1). By its use one secures better growth of *Nitrosomonas* and permits the heterotrophic colonies to develop sufficiently to be seen and avoided in the selection of pure colonies to be picked.

Heavy films of sterile calcium carbonate or magnesium carbonate on the surface of sterile plates, to indicate the sites of nitrification (Winogradsky and Winogradsky, 1933), cannot be employed in pure culture isolation. Although the carbonate film may appear to be dry, it holds capillary water and permits spreading of the various bacteria. Every attempt to obtain pure cultures in these films was unsuccessful. Furthermore, decomposition or etching of these films results only from the rapid production of nitrous acid by large colonies or colony clumps, while small colonies do not produce perceptible etching. Many colonies

which could be seen easily on the clear plates are obscured by the carbonate.

Suspensions suitable for plating

Since the early work of Winogradsky, investigators in this field have recognized the importance of increasing the ratio of *Nitrosomonas* to contaminants in cultures started from soil, in order that pure cultures might be obtained by dilution, by picking single cells, or by plating. In this study various methods of producing cultures for plating have been compared and considerable attention has been given to the problem of obtaining suspensions in which the ratios are suitable for the isolation of pure colonies. The results summarized below are based on work with 9 samples of garden soil from the District of Columbia and Arlington County, Virginia.

Enrichment cultures were started by adding various amounts of soil (1, 0.1 and 0.01 gram) to tubes of the liquid medium. Larger amounts of soil were detrimental to the initiation of oxidation. Serial transfers were then made from the tube first showing a 2+ reaction for nitrite (one loop of culture tested in a spot plate with Trommsdorf's reagent). After nitrification had occurred in the second or third tube of the series, little was gained by further transfers because of the continued luxuriant growth of several of the contaminating species. In our experience the sodium-caseinate agar (Fred and Waksman, 1928) plate count of these contaminants rose to levels of 10 to 100 million viable cells per cubic centimeter within 2 or 3 days following the inoculation of new tubes with enrichment culture. The proliferation of the ammonia-oxidizing bacteria was so slow that several weeks of incubation, and fresh additions of ammonium sulphate, were required to produce counts of a hundred thousand to several million organisms per cubic centimeter. The numbers of nitrifying bacteria were ascertained by counting colonies on silica gel plates. These figures were checked by determining the highest dilutions of the enrichment cultures capable of causing nitrification in the liquid medium. These methods were laborious and the results were obtained, of course, after too great a delay to serve as a useful index of suitable plating ratios.

Since these experiments had shown also that crude cultures containing about one million viable cells of *Nitrosomonas* per cubic centimeter were necessary in order to produce pure colonies, it was desirable to find some method for learning more quickly and easily when such ratios had been reached. A correlation between the count of viable *Nitrosomonas* and the production of nitrite was sought. In agreement with the recommendation of Winogradsky and Winogradsky (1933), it was found that the nitrite in the cultures could be estimated semi-quantitatively by adding one drop of each of the Trommsdorf reagents to 0.1 cc. of culture or culture dilutions in a spot plate. The reliability of this method was checked by observing the intensity of the Trommsdorf test in various dilutions of the culture medium to which a known

TABLE 1

MILLIGRAMS OF NO_2^- NITROGEN PER CUBIC CENTIMETER	STRENGTH OF REACTION:	
	Recorded	Description
0.0002	−	No color in first minute
0.0005	1+	Immediate color; very pale blue
0.0010	2+	Immediate color; deep blue
0.0020	3+	Immediate color; purple

amount of sodium nitrite had been added. Table 1 shows the values thus determined.

Simultaneous colony counts and nitrite determinations were made in enrichment cultures. They indicated that cultures containing 0.1 mgm. of nitrite nitrogen per cubic centimeter would produce approximately 1 million colonies per cubic centimeter. The $1:10^4$, $1:10^5$ and $1:10^6$ dilutions of such cultures were plated. The plating of lower dilutions of enrichment cultures proved to be futile. Fifteen pure cultures were picked from plates inoculated with the $1:10^4$ dilution, 40 were picked from those of the $1:10^5$ dilution and 7 from the $1:10^6$ dilution plates. These pure cultures represent only the picking of sufficient colonies from each experiment to make sure of the results. Undoubtedly many more pure cultures could have been secured had more colonies been picked. Although this procedure has con-

sistently permitted pure culture isolation from crude enrichment cultures plated under the conditions described, the slowness of the method, together with the attention required in supplying the ammonia and carbonate and in following the nitrite accumulation, made it undesirable as a general procedure.

A more convenient and rapid means of obtaining suspensions with suitable ratios of *Nitrosomonas* was afforded by picking and emulsifying contaminated colonies or masses of growth from plates which had been inoculated with soil. Various methods of obtaining these colonies from soil have been compared on the silica gel plates recommended (called "clear" plates) and on similar plates covered with films of carbonate according to the directions given by Winogradsky (called "filmed" plates). Plates of each type were inoculated with from 1 to 20 soil particles (about 1 mm. in diameter), with small amounts of pulverized soil sifted onto the plates through fine cheese cloth and with increasing dilutions of 10 per cent soil suspensions.

On the "filmed" plates, inoculations with soil particles or with sifted soil gave better results than the diluted soil suspensions. Sites showing complete etching of the carbonate film were apparent after 10 to 30 days at 28°C., depending on the soil used and the amount employed. In these cleared areas there were no individual colonies of *Nitrosomonas* but rather a mass of growth composed of nitrifying bacteria and contaminants. Since the ratio of *Nitrosomonas* to contaminants in these sites was barely favorable, it was important to avoid including soil with the material collected for suspension. In most cases, the amount of growth which could be collected on an inoculating needle, when suspended in the liquid medium, required further dilutions of $1:10^3$, $1:10^4$, and $1:10^5$ for the production of pure cultures on the final set of plates.

On "clear" plates the dilutions of soil suspension deposited débris so uniformly that the growth of *Nitrosomonas* was obscured. On "clear" plates inoculated with soil particles or with finely sifted soil, the ammonia-oxidizing bacteria grew around the margin of soil particles as well-defined "rims," or as complex masses of colonies; between the soil particles they grew as minute

colonies and, occasionally, as clear-cut colonies (see figs. 1 to 3). When the soil particles were removed from the plates after nitrification was detected with Trommsdorf reagent, the area of the gel beneath the soil particles also developed large numbers of colonies (see fig. 4). The large colonies, "rims," and masses of growth on the clear plates could be recognized with the hand lens or colony microscope after incubation for 15 to 40 days. In order to avoid possible confusion of the individual colonies with grains of sand on the surface of the plates and for the sake of selecting sites of growth which are most suitable for picking, such areas were examined also with the low power of the compound microscope. The clear-cut colonies, the colony masses around minute particles of soil, or the growth around larger soil particles (when collected with care), contained suitable proportions of *Nitrosomonas* and consistently permitted the isolation of pure cultures. The amounts of material and the dilutions recommended above should be used for the inoculation of the second set of plates.

The degree of etching on the "filmed" plates inoculated with soil, or the amount of visible growth on the "clear" plates, was never proportional to the amount of nitrite formed. It has been noted that examination of the growth on the clear plates revealed numerous colonies which remained small. These minute colonies probably originated from the spreading of motile *Nitrosomonas* cells in films of liquid drawn to the surface of the medium by the soil particles. Such colonies undoubtedly occurred on the filmed plates as well. It was not clear whether the enormous number of these small colonies or the multiplication of *Nitrosomonas* chiefly in the soil particles inhibited the rapid growth of larger visible colonies.

Accordingly a study was next made of several methods by which ammonia-oxidizing bacteria might be separated from soil prior to the first inoculations on "clear" plates. One-half cubic centimeter portions of supernatant fluids obtained from fresh soil suspensions, from suspensions of soil which had been enriched on the solid medium, and from liquid cultures inoculated with soil were plated directly and in dilutions of 1:10 and 1:100. The supernatant fluids from soil suspensions were prepared by shaking

2 grams of soil in 20 cc. of the liquid medium and by centrifuging this suspension at 2000 r.p.m. for 2 minutes; the undiluted inoculum sometimes produced large colonies on the solid medium in 3 weeks, but the results were often negative when soils with low counts were used. The enrichment on plates was carried out by placing a thin layer of soil on the solid medium, inverting the plate to remove all the soil which had not been dampened sufficiently to stick to the gel, and incubating until the jelly gave a 1+ or 2+ reaction for nitrite. At that time, 5 loops of the soil were placed in the liquid medium, the tube was shaken and centrifuged and the supernatant fluid was plated. The liquid cultures inoculated with soil were started as for serial enrichments. The undisturbed supernatant fluid from the tube which first gave a 2+ nitrite reaction was placed on one set of plates. The tube was then shaken and centrifuged and a duplicate set of plates was made with the supernatant fluid. The supernatant fluids obtained from the soil enriched on the solid medium, or from the liquid soil culture produced large and easily recognizable colonies on the plates by the end of three weeks of incubation (see fig. 5).

The principle of separating the organisms from the soil prior to the first plating proved to be superior to all others studied. By this method, the first pickable colonies were obtained more quickly than by placing soil particles directly on the plates; dilution of the supernatant fluids prevented an excessive number of colonies from interfering with rapid growth; and the colonies which developed on the plates were recognized with ease and certainty under a hand lens or colony microscope. When picked for replating, these colonies afforded very favorable ratios of ammonia-oxidizing bacteria. They should be suspended and further diluted to $1:10^2$, $1:10^3$ and $1:10^4$ for the inoculation of the final set of plates.

Inoculation of plates

Since colonies of *Nitrosomonas* are not always readily disintegrated, all suspensions for the inoculation of the final set of plates were prepared by incubating the colonies or the material collected from the first plates in a few drops of the liquid medium for

one hour. The material was then rubbed vigorously with an inoculating needle or sterile stirring rod before the remainder of the medium was added. These suspensions were thoroughly shaken and further diluted as indicated above.

It was found that inoculation of the medium by streaking should be avoided, because the slightest pressure on the surface of the gel caused the liberation of a film of moisture in which the contaminating organisms moved about. The entire plate was thus covered with a layer of diffuse growth which could scarcely be detected with the high power of the microscope under reduced illumination.

The method employed consisted in placing 0.5 cc. of each of the recommended dilutions of the enrichment cultures or of the colony suspensions near the edge of duplicate plates. This inoculum was spread over the surface by holding the closed plate in a horizontal position and causing it to oscillate through a short arc by a rapid side to side movement of the wrist. The suspension was thus moved evenly across the plate and did not run in streaks.

Incubation of plates

The length of the incubation period and the importance of employing media with a dry surface made it necessary to conserve the moisture in the plates if the colonies had not been picked by the end of the second or third week of incubation. Cracking of the silica gels was largely avoided by placing the plates in screw-top "Bokar" coffee cans. The time at which the cans should be used may be determined by observing when water of condensation no longer appears on the lids and perceptible cracks first occur in the medium. No device for the conservation of moisture should be employed while there is any possibility of the medium becoming moist on the surface.

The colonies

When examined with the low-power objective of the microscope, small colonies of *Nitrosomonas* appeared as shining blue or green discs. As they grew older and increased in size, the color gradually changed to brown. The colonies became opaque, coarsely

granular and highly convex. The character of the surface, the regularity of the margin and especially the consistency of the colony were controlled by the conditions of growth. Boltjes has shown that Winogradsky's attempt to establish a new species in the group on the basis of "hard" and "soft" colony types is invalid. In the present study, the colonies produced on the silica gel medium by individual strains of *Nitrosomonas* showed all degrees of hardness and resistance to emulsification. A dry, alkaline medium favored the "hard" types and these were practically never seen on plates of the optimal pH and moisture content.

The convexity and opacity of the well-developed colonies give them a "pearl-like" appearance which facilitates their recognition with the hand lens or colony microscope through the bottom of closed Petri dishes..

Selection of the colonies and picking pure cultures

When the colonies had reached a diameter of about 0.25 mm., they were counted with a hand lens or colony microscope and the position of each was marked with a small circle on the bottom of the Petri dish. Each colony (on plates showing from 1 to 60 colonies) was then carefully examined through the bottom of the plate by the low power objective with the illumination adjusted so that the faintest growth of contaminants could be detected. All colonies which were not homogeneous or which showed pale translucent growth about their margins were avoided because such colonies are always contaminated. Five colonies which appeared free from contamination were picked from each plate and transferred to the liquid medium. In a typical picking of 125 colonies selected in this manner 86 were found to be pure. The picking was carried out with sterile capillary pipettes which were repeatedly drawn out to new tips in a microburner as the picking progressed. The pipettes may have a small opening for picking the softer colonies because these need only to be touched with the pipette and the cells will be drawn in by capillary attraction. With the harder colonies, the whole colony can be drawn into a pipette with a larger opening. The tip of the pipette

containing the culture was broken off in the tube of the liquid medium to be inoculated.

The tubes containing the picked colonies were incubated at 28°C. until a strong test for nitrite (2+ or 3+) was obtained with Trommsdorf's reagent. One loopful of the culture was then transferred to a second tube of the medium. The remainder of the medium in the first tube was tested *in toto* for the presence of heterotrophic organisms by adding 2 cc. of extract broth which had been prepared three times as concentrated as the usual formula (Difco). No final conclusion concerning the absence of growth in the organic medium should be made until these tubes have been incubated for at least 3 weeks, because the appearance of growth is often delayed.

The pure cultures were maintained in the liquid medium. They were allowed to grow at 28°C. only long enough to develop a 2+ nitrite reaction and were then stored in the ice box for periods of approximately 3 months between transfers. Previous to making new transfers, the tubes were removed from the ice box and incubated at 28°C. until growth had again become active.

DISCUSSION

Although the pure culture isolation of *Nitrosomonas* is more time-consuming and less convenient than the procedures commonly required for the majority of bacteria, it has been found to be practicable and reliable when due regard is given to the various difficulties involved. Each of the steps recommended was carried out with different soil samples, silicates, batches of medium, lots of chemicals, and types of bacterial suspensions until it was felt that the procedure could be employed satisfactorily by any competent bacteriologist. Each of the methods outlined, and perhaps others, for obtaining suitable ratios for plating should receive further trials with a wider variety of soils.

Since replating of colonies was found to be more rapid and convenient than the production of suitable serial enrichment cultures in the liquid medium, only the production of impure colonies to be replated will be discussed further. The etched areas on "filmed" plates inoculated with soil have the single advantage of

assisting the novice in the selection of material to be replated. In all other respects these "filmed" plates are less useful than the "clear" ones, especially in view of the fact that the number of contaminating organisms is high in the etched areas. The "clear" plates inoculated with soil produce individual colonies or masses of growth containing more favorable proportions of ammonia-oxidizing bacteria. However, in this case, experience is required to recognize the growth among the soil particles. Several photographs have been included for assistance in this connection.

The procedure which is recommended involves the separation of the ammonia-oxidizing bacteria from soil particles and débris prior to making the first set of "clear" plates. Inoculation of these plates with supernatant fluids from suspensions of soil which have been enriched on the solid medium, or in the liquid medium, quickly produces easily recognized colonies and avoids the disadvantages which arise from placing soil particles directly on the plates. When these colonies are suspended, diluted and replated, pure cultures are produced in less time and with less difficulty than by any other procedure employed.

The pitfalls involved in any assumption that one can depart from the highest standards of aseptic technique in either the isolation or the study of nitrifying bacteria are illustrated by the tardy development of pure culture methods in this field and by the recent erroneous establishment of the genus *Nitrosocystis* by Winogradsky and Winogradsky (1933). Boltjes readily demonstrated with his pure cultures that the organisms designated as *Nitrosocystis* are in reality only "hard" colony types of *Nitrosomonas*. Our own observations on this point are completely in accord with those of Boltjes.

Several misleading impressions which might originate from the recent paper by Winogradsky and Winogradsky (1933) should be clarified. It is stated that, "Il est à remarquer une fois pour toutes, que toutes ces opérations ne présentent aucun risque de contamination, l'air étant toujours dépourvu de germes nitrificàteurs et les germes banaux étant absolument négligeables dans le cas. Les opérations de stérilization, particulièrement à l'auto-

clave, classiques en microbiologie, peuvent donc être de beaucoup réduites sans crainte de brouiller le résultat." The serial transfer of growth or colonies from the etched sites on non-sterilized "filmed" plates is discussed under the headings of "Différenciation et isolement des souches" and "Repiquage des souches et culture pure." Culture purity was tested as follows: "Pour reconnaître si la culture est pure ou presque, il suffit de soumettre à un examen microscopique soigneux quelques colonies parues. . . . Si l'on ne trouve là rien d'étranger, la culture est considérée comme pure." In this connection it should be noted that Winogradsky does not actually regard these cultures as pure in the strict sense of the word, but merely as suitable for studies on nitrification (Winogradsky, 1935). These quotations are given in order to point out the unfortunate and indiscriminate usage of the terms "isolation" and "pure culture" in the paper and in order to emphasize the fact that Winogradsky himself is not under misapprehension concerning the contaminated nature of the cultures with which he has been working.

Nitrification tests of all the heterotrophic organisms frequently encountered in this study showed their inability to oxidize ammonia to nitrite and supported Winogradsky's classical demonstration of the autotrophic nature of nitrification.

The results of this study are in agreement with the following important points emphasized by Boltjes: the advantage of using tap water in the medium, the impossibility of obtaining pure cultures on streaked plates, the identity of the "hard" and "soft" colony types, and the fact that copper carbonate in enrichment cultures (see Nelson, 1931) has no selective action favorable to *Nitrosomonas*.

When the harder types of colonies herein described were suspended in hanging drops and examined under the oil immersion objective, the colonies appeared to imbibe water, the cells inside set up a violent agitation and soon swarmed through a rupture in the colony wall. Outside of the areas in which they were crowded most densely they were seen as pairs of cells showing remarkable motility. Soft colonies which were transferred to the hanging drop on bits of gel behaved in much the same way except that the

motile cells swarmed from the entire surface of the colony. Gram stains of the cells liberated from these colonies have consistently revealed small Gram-negative diplococci.

In view of the present inadequate classification of autotrophic bacteria causing the oxidation of ammonia, the name *Nitrosomonas* has been used in this paper only as a term of convenience.

SUMMARY AND CONCLUSIONS

The isolation of pure cultures of ammonia-oxidizing bacteria was found to be entirely practical and consistently successful, provided:

(a) That the silica jelly medium was prepared with a stable pH and appropriate nutrients, was autoclaved for sterilization, and possessed a dry surface, and

(b) That the suspension to be plated contained suitable proportions of ammonia-oxidizing bacteria.

The method for preparing the medium has been given and several means of obtaining suitable suspensions have been developed. No special apparatus was needed to prepare the medium or to pick the colonies.

REFERENCES

BOLTJES, K. Y. J. 1935 Arch. f. Mikrobiol., **6**, 79–138.

BONAZZI, A. 1919 Bot. Gaz., **68**, 194–206.

HANKS, J. H., AND WEINTRAUB, R. L. 1936 Jour. Bact., **32**, 659–671.

HEUBÜLT, J. 1928 Planta, **8**, 398–422.

FRED, E. B., AND WAKSMAN, S. A. 1928 Laboratory Manual of General Microbiology, McGraw-Hill Book Company, New York City.

NELSON, D. H. 1931 Zentralbl. f. Bakt., II Abt., **83**, 280–311.

RUBENTSCHIK, L. 1929 Zentralbl. f. Bakt., II Abt., **77**, 1–19.

WINOGRADSKY, S. 1890 Ann. de l'Inst. Past., **4**, 213–231.

WINOGRADSKY, S., AND WINOGRADSKY, H. 1933 Ann. de l'Inst. Past., **50**, 350–434.

WINOGRADSKY, S. 1935 Personal communication.

The figures show growth of the ammonia-oxidizing bacteria on the solid medium at 21 days; all cultures were started at the same time from the same soil.

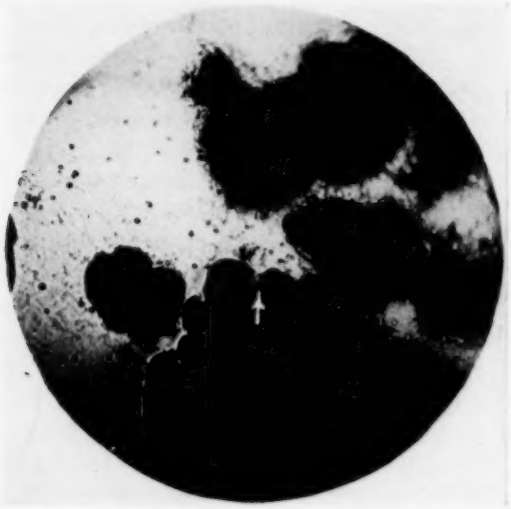


FIG. 1. "RIMS" OR HALOS OF GROWTH AROUND THE EDGE OF A SOIL PARTICLE. MINUTE COLONIES BETWEEN SOIL PARTICLES. (×55)

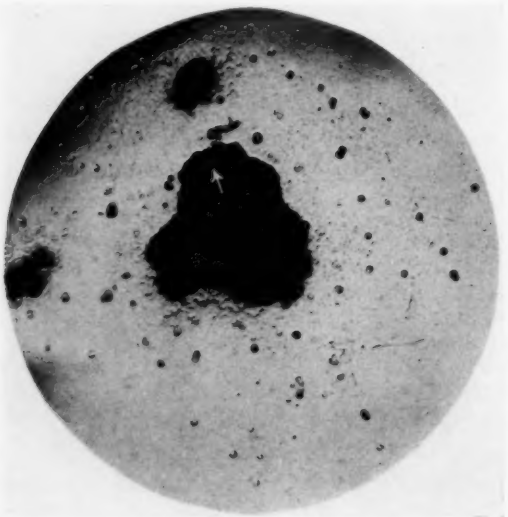


FIG. 2. OUTGROWTH FROM BENEATH A SMALL BIT OF SPRINKLED SOIL. MINUTE COLONIES ALSO SHOWN. (×55)

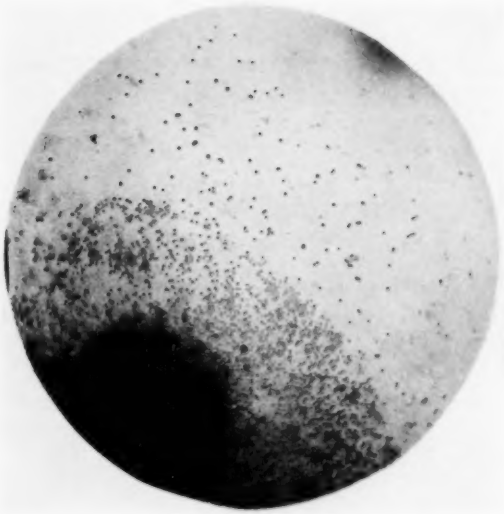


FIG. 3. MASSES OF SMALL COLONIES AROUND A SOIL PARTICLE. (×55)

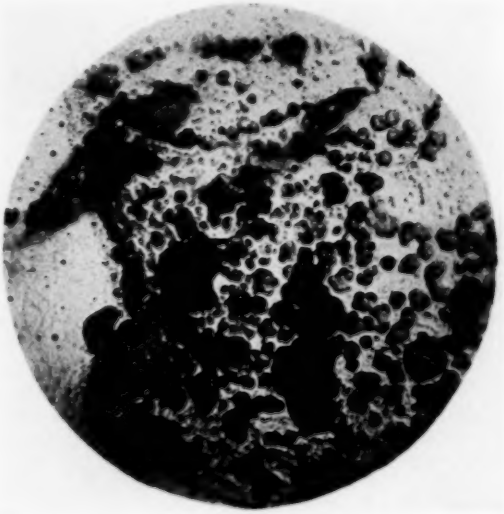


FIG. 4. COLONIES IN A SITE FROM WHICH A SOIL PARTICLE WAS REMOVED AT 7 DAYS. (×55)

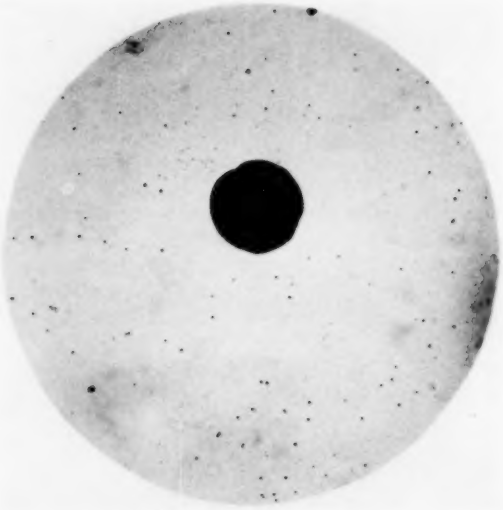


FIG. 5. COLONY PRODUCED BY PLATING SUPERNATANT FLUID FROM SOIL WHICH WAS ENRICHED FOR 2 DAYS ON THE SOLID MEDIUM

These colonies are much easier to recognize and pick than are the growths on soil plates. (×55)

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA BRANCH

COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, OCTOBER 27, 1936

THE INCIDENCE OF TUBERCULOUS INFECTION IN AMERICAN COLLEGES. *Esmond R. Long*, Henry Phipps Institute, University of Pennsylvania, Philadelphia.

The results of about 18,000 accurately completed tuberculin tests with Purified Protein Derivative in twenty colleges for the year 1935–36 were presented. Relatively high rates (40–60 per cent) were found among entering college students in the East and far West and low rates (20–30 per cent) in the central states. Excessive rates were found in colleges in regions noted as resorts for tuberculous patients. The incidence of positive tuberculin reaction was higher in women than in men in the same age groups. The figures were believed to reflect the incidence of tuberculous infection in these regions, since the majority of the entering students in the colleges concerned were residents of the region in which a given college was situated.

CHEMICAL AND IMMUNOLOGICAL RELATIONSHIPS BETWEEN THE PROTEINS FROM DIFFERENT ACID-FAST BACILLI. *Florence B. Seibert*, Henry Phipps Institute, University of Pennsylvania, Philadelphia.

A SEROLOGICAL COMPARISON OF THE PROTEINS OF VARIOUS STRAINS OF LEPROSY BACILLI AND OTHER ACID-FAST BACTERIA. *Howard J. Henderson*, Henry Phipps Institute, University of Pennsylvania, Philadelphia.

The purpose of this study was to determine the antigenic relationship between the different strains of so-called leprosy bacilli and other acid-fast organisms. Use was made of the precipitin test upon the purified proteins isolated from the different micro-organisms. It was found that with few exceptions the specific antigen gave the highest titre. By use of this precipitin method the specificity of any acid-fast strain could be determined. Definite antigenic relationships were found between different strains, which made it possible to place them in certain groups. One large outstanding group was the leprosy group, which included thirteen out of the sixteen cultures isolated from leprosy patients. There were other smaller groups containing fewer acid-fast strains. Some of the organisms fell into two different groups, indicating a border-line affinity. These qualitative differences indicate that further quantitative studies should be undertaken.

FURTHUR STUDIES ON THE MECHANISM OF IMMUNITY TO TUBERCULOSIS. *Max B. Lurie*, Henry Phipps Institute, University of Pennsylvania, Philadelphia.

If virulent tubercle bacilli incor-

porated in melted agar are injected subcutaneously into normal and immunized rabbits the microörganisms fail to grow in the acellular agar of the sensitized animal, while they grow unhindered in that of the normal animal. The following experiment seems to demonstrate this extracellular factor. If silk bags are impregnated with a collodion solution which permits the passage of proteins but prevents the entrance of cells, and if such bags containing tubercle bacilli are placed in the peritoneal cavity of rabbits, the body fluids of normal animals support the growth of the bacilli, whereas the body fluids which penetrate the bags *in vivo* from tuberculous animals do not.

There is a marked acceleration of the mobilization of mononuclear phagocytes at the site of reinfection as compared with that at the site of primary infection. This is not due to the slightly lower pH which develops at the site of reinfection. Nor is this due to a greater permeability of the blood vessels of tuberculous rabbits resulting from irritation, as compared with that of normal animals. Simultaneous antibody titers of the blood and exudates of tuberculous and normal animals do not differ materially. The protein concentration of the exudate induced by aleuronat in tuberculous guinea pigs is higher than that of normal guinea pigs. Guinea pigs also differ from rabbits in the greater capacity of their inflammatory reaction to fix the bacilli of reinfection. Is this difference between the two species due to differences in permeability?

CENTRAL PENNSYLVANIA BRANCH

PENNSYLVANIA STATE COLLEGE, OCTOBER 10, 1936

BRUCELLOSIS. *Henry F. Hunt and Mary E. Knoll*, Department of Pathology, Geissinger Memorial Hospital, Danville, Pa., and Department of Bacteriology, Bucknell University, Lewisburg, Pa.

The incidence of brucellosis is steadily on the increase in the United States. The Public Health Service reports a total of 1788 cases for 1933 and 2017 cases in 1934. In 1934 and 1935, 162 cases were reported to the Pennsylvania Department of Health.

The symptoms are usually quite characteristic by the time the patient seeks medical aid. The onset may be insidious or abrupt and the symptoms may vary in intensity. Weakness, sweating and generalized aches and pains accompanied by fever are the most common manifestations. A positive diagnosis can be made upon the isolation of the organism from a blood culture, the exudate of an involved part, or by use of the agglutination test or the intradermal test.

The presence of native agglutinins in the blood serum may often be misleading and a positive agglutination test must be interpreted in the light of the clinical findings. One thousand samples of blood serum were examined for the presence of agglutinins for the *Brucella* group and 89 or 8.9 per cent gave significant findings. Of this group only two patients had definite clinical brucellosis.

Various methods of treatment have been used. The twenty cases observed were all treated by the intravenous administration of mixed typhoid bacterin. In every instance the results were satisfactory and on follow-up examination nineteen showed negative agglutination reactions for brucellosis.

A STUDY OF AN ATYPICAL CASE OF TYPHOID FEVER. *Arthur Brumbaugh*, Mercy Hospital, Altoona, Pa.

A STUDY OF CONFIRMATORY PROCEDURES IN THE BACTERIOLOGICAL ANALYSIS OF WATER. *Michael A. Farrell*, The Pennsylvania State College, State College, Pa.

In an investigation to learn whether the time interval often necessary to confirm the presumptive evidence of pollution can be shortened, seven presumptive test media were used alone as complete confirmatory procedures in comparison with Standard Methods Procedure. The results of examining ninety-four water samples from surface streams as well as deep and shallow wells indicated that the time interval for confirming positive and doubtful presumptive tests could be appreciably shortened without lessening the efficiency of the analysis. Eccentric strains of the *Escherichia-Aerobacter* group deserve special study before one of these presumptive test media is recommended.